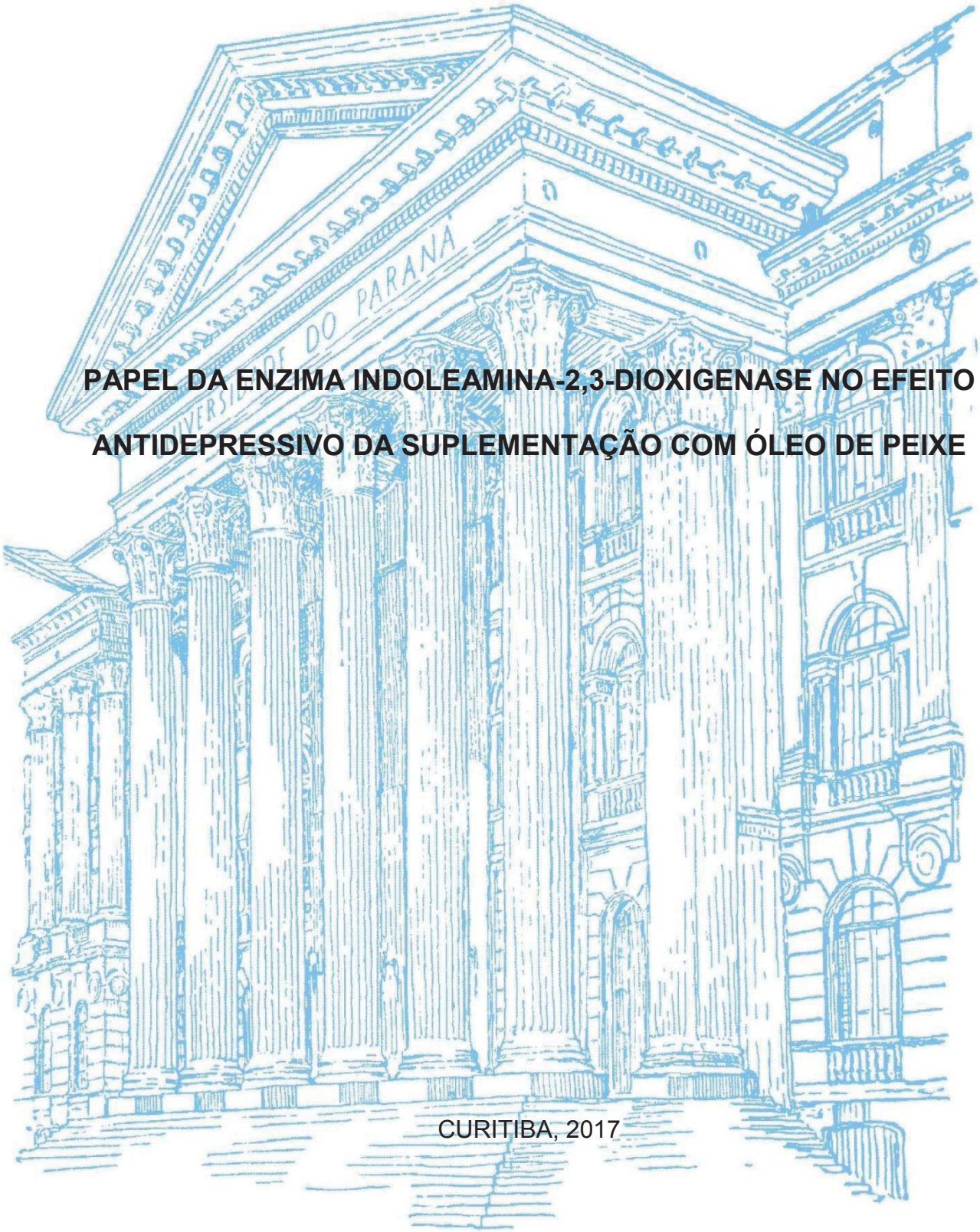
**UNIVERSIDADE FEDERAL DO PARANÁ**

BRUNO CARABELLI




# PAPEL DA ENZIMA INDOLEAMINA-2,3-DIOXIGENASE NO EFEITO ANTIDEPRESSIVO DA SUPLEMENTAÇÃO COM ÓLEO DE PEIXE

CURITIBA, 2017

BRUNO CARABELLI

# PAPEL DA ENZIMA INDOLEAMINA-2,3-DIOXIGENASE NO EFEITO ANTIDEPRESSIVO DA SUPLEMENTAÇÃO COM ÓLEO DE PEIXE

Tese apresentada como requisito para obtenção do grau de doutor em Fisiologia. Programa de Pós-Graduação em Fisiologia, Setor de Ciências Biológicas, Universidade Federal do Paraná

**Orientadora**: Profa. Dra. Anete Curte Ferraz
**Coorientadora**: Prof. Dra. Janaína M. Zanoveli

**CURITIBA, 2017**

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Departamento de Fisiologia
Programa de Pós-Graduação em Fisiologia

# D E C L A R A Ç Ã O

Declaramos para os devidos fins que **BRUNO CARABELLI** no dia 11 de dezembro de dois mil e dezessete, no Setor de Ciências Biológicas (UFPR) defendeu sua Tese de Doutorado em Fisiologia, intitulada: "PAPEL DA ENZIMA INDOLEAMINA-2,3-DIOXYGENASE NO EFEITO ANTIDEPRESSIVO DA SUPLEMENTAÇÃO COM ÓLEO DE PEIXE", com a banca examinadora constituída pelos Professores: Profa. Dra. Célia Regina Ambiel (Departamento de Fisiologia da Universidade Estadual de Maringá - UEM), Professora Doutora Joice Maria da Cunha (Departamento de Farmacologia da Universidade Federal do Paraná - UFPR), Professora Doutora Maria Aparecida Barbato Frazao Vital (Departamento de Farmacologia da Universidade Federal do Paraná - UFPR), Professora Doutora Fabíola Iagher (Departamento de Fisiologia da Universidade Federal do Paraná - UFPR) e Professora Doutora Anete Curte Ferraz (Departamento de Fisiologia da Universidade Federal do Paraná - UFPR) como orientadora e presidente da Banca Examinadora. Tendo sido **Aprovado**, sendo que a emissão do diploma da mesma ficará condicionada à implementação das correções sugeridas pelos membros da banca examinadora e ao cumprimento integral das exigências estabelecidas no Art. 61º do Regimento interno deste Programa de Pós-Graduação, bem como do item III do artigo 80 da resolução 65/09 do CEPE-UFPR.

Esta Declaração tem validade por 60(sessenta) dias, a partir da data de emissão da mesma.

Curitiba, 11 de dezembro de 2017.

Fernando Augusto Lavezzo Dias
Coordenador do Programa de Pós-Graduação em Fisiologia

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UFPR Biológicas

# Ata da Defesa de Tese de Doutorado de BRUNO CARABELLI

Aos onze dias do mês de dezembro do ano de dois mil e dezessete, foi realizada no auditório do Departamento de Fisiologia no Setor de Ciências Biológicas da Universidade Federal do Paraná, a defesa de tese do doutorando **BRUNO CARABELLI,** intitulada **"PAPEL DA ENZIMA INDOLEAMINA-2,3-DIOXYGENASE NO EFEITO ANTIDEPRESSIVO DA SUPLEMENTAÇÃO COM ÓLEO DE PEIXE".** A abertura teve início às 08h30min pela Presidente da Banca Examinadora e Orientadora do candidato, Professora Doutora Anete Curte Ferraz. A Presidente apresentou ao público presente os membros da banca examinadora e logo passou à palavra ao aluno, para que fizesse uma apresentação sucinta de sua tese. Após a explanação oral, a Professora Doutora Anete Curte Ferraz passou à palavra a primeira examinadora, Professora Doutora Célia Regina Ambiel do departamento de Fisiologia da Universidade Estadual de Maringá (UEM). Na sequência, passou à palavra a segunda examinadora, Professora Doutora Joice da Cunha Maria da Cunha do Departamento de Farmacologia da UFPR. Em seguida Passou à palavra a terceira examinadora Professora Doutora Maria Aparecida Barbato Frazao Vital do Departamento de Farmacologia da UFPR e por último passou a palavra à quarta examinadora Professora Doutora Fabíola Iagher, do Departamento de Fisiologia da UFPR. O aluno respondeu as perguntas dos examinadores e se posicionou frente às críticas. Findas as arguições pelos demais membros da banca, a Presidente, Professora Doutora Anete Curte Ferraz fez uma rápida apreciação das conclusões mais importantes dos debates realizados e comunicou que a Banca Examinadora iria reunir-se em sessão secreta para discussão e atribuição dos conceitos. Os trabalhos foram interrompidos por cinco minutos. Após haver analisado o referido trabalho e argüido o candidato, os membros da banca examinadora reunidos em sessão secreta deliberaram pela "aprovação", habilitando-o ao título de Doutor em Fisiologia, condicionada à implementação das correções sugeridas pelos membros da banca examinadora e ao cumprimento integral das exigências estabelecidas no Art. 59° do Regimento interno deste Programa de Pós-Graduação e no no Art. 61° do Regimento interno deste Programa de Pós-Graduação, bem como do item III do artigo 80 da resolução 65/09 do CEPE-UFPR. Eu, Professora Doutora Anete Curte Ferraz, Presidente da Banca Examinadora lavrei a presente ata, da qual assino juntamente com os senhores examinadores.

Curitiba, 11 de dezembro de dois mil e dezessete.

**Profa. Dra. Célia Regina Ambiel**
UEM - Membro Titular

**Profa. Dra. Joice Maria da Cunha**
UFPR - Membro Titular

**Profa. Dra. Maria Barbato Vital**
UFPR - Membro Titular

**Profa. Dra. Fabíola Iagher**
UFPR - Membro Titular

**Profa. Dra. Anete Curte Ferraz**
UFPR - Orientadora e Presidente da Banca Examinadora

Ministério da Educação
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# PARECER

Os abaixo-assinados, membros da Banca Examinadora da Defesa de Tese de Doutorado, a qual se submeteu **BRUNO CARABELLI** para fins de obter o título de Doutor em Fisiologia pela Universidade Federal do Paraná, são de parecer à aprovação (aprovação/reprovação) da acadêmica.

A obtenção do título está condicionada à implementação das correções sugeridas pelos membros da banca examinadora e ao cumprimento integral das exigências estabelecidas no Regimento interno deste Programa de Pós-Graduação, bem como do item III do artigo 80 da resolução 65/09 do CEPE-UFPR.

Curitiba, 11 de dezembro de 2017.

| Parecer (Aprovada/Reprovada) | Nome | Assinatura |
|---|---|---|
| Aprovado | **Profa. Dra. Célia Regina Ambiel**<br>UEM - Membro Titular | |
| APROVADO | **Profa. Dra. Joice Maria da Cunha**<br>UFPR - Membro Titular | |
| Aprovado | **Profa. Dra. Maria Barbato Vital**<br>UFPR - Membro Titular | |
| Aprovado | **Profa. Dra. Fabíola Iagher**<br>UFPR - Membro Titular | |
| Aprovado | **Profa. Dra. Anete Curte Ferraz**<br>UFPR - Membro Titular | |

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## RESUMO

A depressão é uma doença psiquiátrica grave, com uma grande prevalência durante a vida. De acordo com a Organização Mundial da Saúde aproximadamente 320 milhões de pessoas sofrem de depressão em todo o mundo. O tratamento atual é baseado na Teoria Monoaminérgica, porém muitas evidências apontam para o envolvimento da inflamação na patogênese da depressão. Alguns autores mostram que a ligação entre a inflamação e a depressão é feita pela enzima Indoleamina-2,3-dioxygenase (IDO), que é responsável por catabolizar o triptofano dando origem às kinureninas, o que leva à diminuição da síntese de serotonina e promove sintomas depressivos.

Estudos já mostraram que compostos com atividade anti-inflamatória, como os Ácidos Graxos Poli-insaturados Ômega-3, apresentam atividade antidepressiva, e são capazes de aumentar os níveis de serotonina no hipocampo de ratos.

Devido a essas evidências, nós hipotetizamos que o efeito antidepressivo do ômega-3 está relacionado com suas propriedades anti-inflamatórias, levando à inibição da enzima IDO e consequentemente aumentando os níveis de serotonina. Para testar essa hipótese, realizamos dois experimentos independentes, nos quais os animais foram suplementados com óleo de peixe (rico em ômega-3) por 50 dias. Após a suplementação, foi realizada uma única injeção sistêmica com o Lipopolissacarídeo bacteriano (LPS), capaz de induzir uma resposta inflamatória, e 24 horas após os animais foram submetidos aos testes do campo aberto e natação forçada modificado. No primeiro experimento, os animais receberam um pré-tratamento com com o inibidor competitivo da IDO 1-metil-DL-Triptofano (1-MT) e no experimento 2 os animais foram tratados com minociclina, um inibidor indireto dessa enzima. O pré-tratamento com esses inibidores foi feito 23h, 5h e 1h antes dos testes comportamentais. Após os testes, os animais foram ortotanasiados e os hipocampos obtidos para a quantificação de serotonina e seu metabólito ácido 5-hidroxiindolacético (5-HIAA) através da técnica de HPLC e para a expressão da IDO através do western blot. A injeção com LPS foi capaz de induzir um comportamento tipo-depressivo, que foi bloqueado pelo 1-MT, pela minociclina e pela suplementação. Os animais suplementados apresentaram maior fequência de natação comparados aos animais tratados com 1-MT e minociclina. A suplementação e a minociclina foram capazes de diminuir a expressão da IDO, sem efeitos adicionais sobre esta enzima quando os tratamentos foram combinados. Como esperado, o 1-MT não foi capaz de diminuir a expressão da IDO, uma vez que esta droga inibe diretamente esta enzima, impedindo apenas sua atividade. Os dados neuroquímicos mostram que o LPS foi capaz de diminuir a concentração de serotonina e aumentar seu turnover, e esses efeitos foram bloqueados pelos compostos anti-inflamatórios minociclina e óleo de peixe, mas não pelo 1-MT. Ainda, a suplementação com óleo de peixe teve efeito antidepressivo relacionado com o aumento de serotonina no hipocampo. É importante destacar que os animais suplementados que receberam apenas injeções de salina apresentaram níveis de serotonina, expressão da IDO e frequência de natação semelhantes aos animais que foram suplementados e receberam os outros tratamentos combinados. Esse fato indica que a inibição da IDO, embora importante, não é a única explicação para o efeito antidepressivo do ômega-3, e que este composto aumenta os níveis de serotonina através de outros mecanismos.



Palavras-chave: depressão, ômega-3, óleo de peixe, inflamação, LPS, Indoleamina-2,3-Dioxigenase, IDO, serotonina.

## ABSTRACT

Depression is a severe psychiatric disease, with a high prevalence during life. Is twice common in women and according to World Health Organization approximately 320 million people suffer from depression worldwide.

The Monoaminergic Theory is the basis of current treatment, but there are many evidence pointing out to the role of inflammation on pathogenesis of depression. Depressed patients have an increased rate of autoimmune disorders, and patients with inflammatory diseases, like cancer, HIV and rheumatoid arthritis have higher rates of depression. In animal models, systemic injection of bacterial endotoxin lipopolysaccharide evokes an acute inflammatory response and 24h after the injection rodents exhibit a depressive-like behavior, seen by higher immobility in the forced swim test. The link between inflammation and depression is made by the enzyme Indoleamine-2,3-dioxygenase (IDO), which is activated by pro-inflammatory cytokines. This enzyme catabolizes tryptophan into kynurenines, decreasing the levels of serotonin and promoting depressive symptoms. Anti-inflammatory compounds, such as Omega-3 Polyunsaturated Fatty Acids, have antidepressant activity, increasing serotonin levels in hippocampus of rats.

Here we hypothesized that this antidepressant effect of omega-3 is due to its anti-inflammatory properties, and the mechanism is through IDO inhibition. In order to test this hypothesis, we performed two independent experiments, using the LPS model in fish-oil supplemented animals. In the first experiment, animals received a pre-treatment with 1-MT and in the experiment 2 they were treated with minocycline. The use of these two inhibitors of IDO was made to test a possible potentiation of omega-3 antidepressant activity.

The supplementation was made for 50 days and LPS was injected 24h before the open field test and the modified forced swimming test. The pretreatment with the inhibitors was made 23h, 5h and 1h before these behavioral tests.

After the tests, the hippocampi were obtained for quantification of serotonin and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) by HPLC and IDO expression by western blot. We found a depressive-like state induced by LPS, which was blocked by 1-MT, Minocycline and fish-oil supplementation. Also, the supplemented animals presented higher swimming behavior compared to 1-MT and Minocycline. Minocycline and fish oil suppressed IDO expression, with no additional effects when these treatments were combined. LPS induced a decrease in serotonin levels and an increase in 5-HIAA/5-HT ratio, both blocked by the anti-inflammatory compounds minocycline and fish oil. The antidepressant-like behavior induced by omega-3 was related to an increase in serotonin levels in hippocampus, compared to all non-supplemented groups. The fact that IDO expression in non-stressed animals is not significant, and the supplemented animals who received only saline presented higher levels of serotonin, support the idea that IDO inhibition is not the only explanation for antidepressant effect of fish-oil supplementation, and this compound increases serotonin through diffrent mechanisms.



Key-words: depression, omega-3, fish oil, inflammation, LPS, Indoleamine-2,3-Dioxygenase, IDO, serotonin.

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# 1 INTRODUÇÃO

## 1.1 Depressão

A depressão é um transtorno psiquiátrico grave, com alta taxa de prevalência durante a vida. É mais comum em mulheres e segundo a Organização Mundial da Saúde aproximadamente 320 milhões de pessoas sofrem de depressão no mundo (ALBERT; FRANCOIS, 2010; WORLD HEALTH ORGANIZATION, 2017).

De acordo com o DSM-V para o diagnóstico da depressão é necessário a presença de sintomas como humor deprimido ou perda de interesse ou prazer por no mínimo 2 semanas, acompanhados de quatro ou mais sintomas como sentimentos de desesperança, desvalia, culpa, desamparo, associados a alterações de apetite e sono, fadiga, retardo ou agitação psicomotora, diminuição do desempenho sexual, dificuldade de concentração e raciocínio e pensamentos recorrentes sobre morte, com ou sem tentativas de suicídio (AMERICAN PSYCHIATRIC ASSOCIATION, 2013).

A teoria predominante sobre as causas da depressão é a teoria das monoaminas, que afirma que a depressão é causada por um déficit na neurotransmissão monoaminérgica no encéfalo (BERTON; NESTLER, 2006; LÓPEZ-MUÑOZ; ALAMO, 2009b; NESTLER et al., 2002; WILLNER; SCHEEL-KRÜGER; BELZUNG, 2013).

Essa teoria foi baseada em observações clínicas sobre o efeito de duas drogas não relacionadas estruturalmente – a iproniazida e a imipramina – que foram desenvolvidas para outras doenças (não-psiquiátricas) e apresentavam potente efeito antidepressivo em seres humanos. Posteriormente descobriu-se que essas drogas aumentam os níveis de serotonina e noradrenalina no encéfalo. Essas descobertas, juntamente com a observação de que a reserpina, um anti-hipertensivo, causava sintomas depressivos por depletar os níveis de monoaminas serviram de suporte à formulação da teoria monoaminérgica (DUMAN; HENINGER; NESTLER, 1997; KRISHNAN; NESTLER, 2008; LÓPEZ-MUÑOZ; ALAMO, 2009a; NESTLER et al., 2002).

Apesar de primeiramente ter sido apontado um papel das catecolaminas (Teoria Catecolaminérgica), evidências posteriores sobre o efeito dos antidepressivos tricíclicos em também aumentar os níveis de serotonina, assim como a

eletroconvulsoterapia, levaram à formulação da Teoria Serotoninérgica da Depressão (BLIER; DE MONTIGNY, 1994; COPPEN, 1967; LAPIN; OXENKRUG, 1969; LÓPEZ-MUÑOZ; ALAMO, 2009b; SCHILDKRAUT, 1965).

Outras evidências também reforçam a importância da neurotransmissão serotoninérgica na depressão. Existe uma forte associação entre baixos níveis do ácido 5-Hidroxiindolacético (5-HIAA), metabólito da serotonina, com a tendência ao suicídio na depressão (ASBERG; TRÄSKMAN; THORÉN, 1976). Há uma relação entre menor expressão de receptores serotoninérgicos 5-$HT_1$ pós-sinápticos, encontrados em áreas como hipocampo, córtex pré-frontal e córtex entorrinal, e sintomas depressivos e risco de suicídio. Estes receptores também se apresentam em menor número e apresentam uma menor afinidade em testes de ligação ao agonista (*binding*), no hipocampo em vítimas de suicídio (CHEETHAM et al., 1990).

Todas essas descobertas levaram ao desenvolvimento de antidepressivos mais modernos, como os Inibidores Seletivos da Recaptação de Serotonina (ISRS).

## 1.2 Depressão e inflamação

A hipótese inflamatória da depressão teve origem em 1991 com o nome de Teoria dos Macrófagos, e afirmava que as citocinas secretadas em excesso pelos macrófagos estariam entre as causas dos sintomas depressivos (SMITH, 1991).

A partir deste trabalho muitas pesquisas tiveram como foco a relação entre o sistema imunológico e a depressão. A observação de que pacientes portadores de doenças onde a inflamação crônica está presente como câncer, hepatite C, HIV, artrite reumatoide e diabetes possuem maiores índices de depressão deixa evidente a relação entre o aumento da inflamação e sintomas depressivos. O tratamento com Interferon-α (IFN-α) em pacientes com hepatite C pode levar à um quadro depressivo grave e essa é uma das evidências mais contundentes de que a inflamação leva a sintomas depressivos (DANTZER et al., 2008; REICHENBERG; GORMAN; DIETERICH, 2005).

O desafio imunológico e a inflamação periférica induzem um estado inflamatório também no sistema nervoso central (SNC), com a secreção de citocinas pró-inflamatórias, sendo capazes de interferir em muitos processos encefálicos, como a neurotransmissão, metabolismo de neurotransmissores e neurogênese (DUNN,

2006; EYRE; BAUNE, 2012; MYINT; KIM, 2003; RUSSO; BARLATI; BOSETTI, 2011; SZELÉNYI, 2001).

A inflamação é uma resposta natural do organismo, que leva a mudanças comportamentais coletivamente chamadas de Comportamento de doença (*Sickess Behavior*), que possui muitos sintomas em comum com a depressão, como letargia, anedonia, perda de apetite, fadiga entre outros (DANTZER et al., 2011a; O'CONNOR et al., 2009b).

A injeção sistêmica com o Lipopolissacarídeo bacteriano (LPS) em roedores leva ao comportamento de doença, que tem seu pico por volta de 6 horas após a injeção e aparentemente desaparece após 24 horas, dando lugar ao comportamento tipo-doentio (DANTZER et al., 2008).

Evidências apontam que a ligação entre a inflamação e a depressão é feita pela enzima Indoleamina-2,3-dioxigenase (IDO). Esta enzima é a primeira de uma via responsável por metabolizar o triptofano, dando origem às quinureninas e é diretamente ativada por citocinas inflamatórias (Figura 1). O triptofano é um aminoácido essencial precursor da serotonina e a ativação da IDO desvia a via em direção a formação de quinureninas, consequentemente diminuindo a formação de serotonina, provocando sintomas depressivos (DANTZER et al., 2011b; MAES et al., 2011b; WICHERS; MAES, 2004).

A inibição da IDO, seja diretamente pelo inibidor competitivo 1-Metiltriptofano (1-MT), ou indiretamente pela injeção de minociclina - um antibiótico pertencente à classe das tetraciclinas com propriedades anti-inflamatórias independentes das antimicrobianas – reverte o comportamento tipo-depressivo provocado pelo LPS, fortalecendo a ideia de que a ativação desta enzima é responsável pelos sintomas depressivos causados pelo aumento da inflamação (O'CONNOR et al., 2009b).

Os sintomas depressivos provocados pelo aumento da inflamação e consequente ativação da IDO podem ser revertidos com antidepressivos, especialmente os ISRS, e também por compostos que possuem efeito antidepressivo e anti-inflamatório, como como os ácidos graxos poli-insaturados da família ômega-3 (AGPIs W-3) (DANG et al., 2017; GALECKI; MOSSAKOWSKA-WÓJCIK; TALAROWSKA, 2017; HANNESTAD; DELLAGIOIA; BLOCH, 2011; SHI et al., 2016).

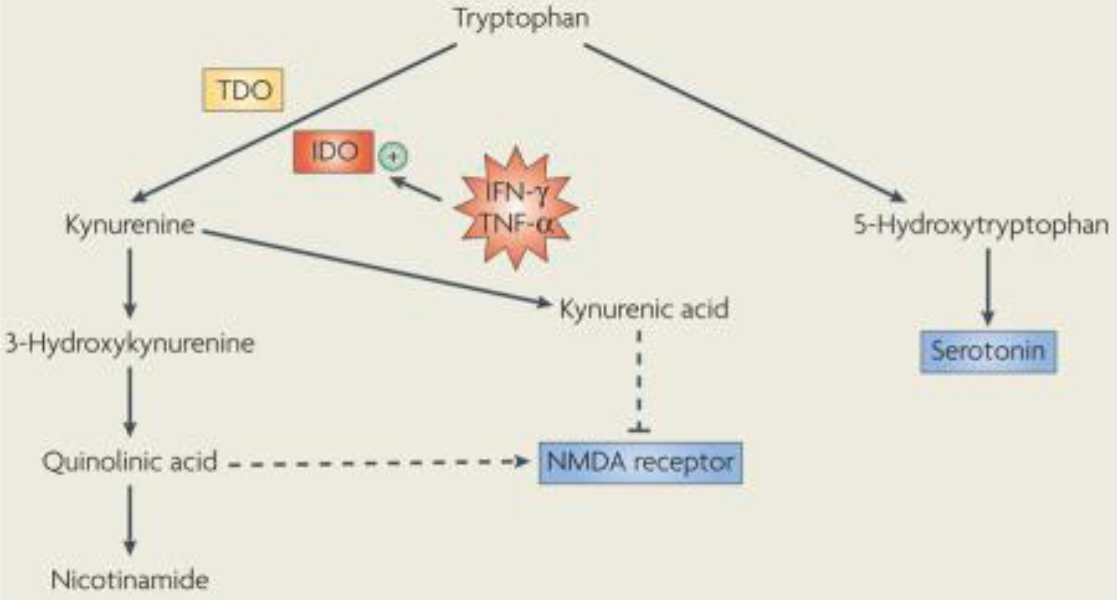


Figura 1 Vias metabólicas do Triptofano: via das quinureninas e via da serotonina (Dantzer et al., 2008).

## 1.3 Depressão e Ácidos Graxos Poli-insaturados Ômega-3

As famílias de ácidos graxos ômega-3 (n-3) e ômega-6 (n-6) são pertencentes à grande familia dos acidos graxos poli-insaturados (AGPIs). São considerados ácidos graxos essenciais, pois os mamíferos não conseguem sintetizá-los, por isso precisam adquirí-los através da dieta (HORROCKS; FAROOQUI, 2004; MCNAMARA, 2016).

O Ácido Linoleico (LA n-6) é o precursor da família n-6, dando origem aos demais ácidos graxos pertencentes ao grupo, entre eles o Ácido Araquidônico (AA) e o Ácido Docosapentaenóico (DPA). O Ácido α-Linolênico (ALA) por sua vez, é o precursor da família n-3, dando origem aos ácidos graxos desta família, que tem como principais representantes os ácidos Eicosapentaenóico (EPA) e Docosahexaenóico (DHA) (Figura 2) (MCNAMARA, 2016; OZIAS; CARLSON; LEVANT, 2007).

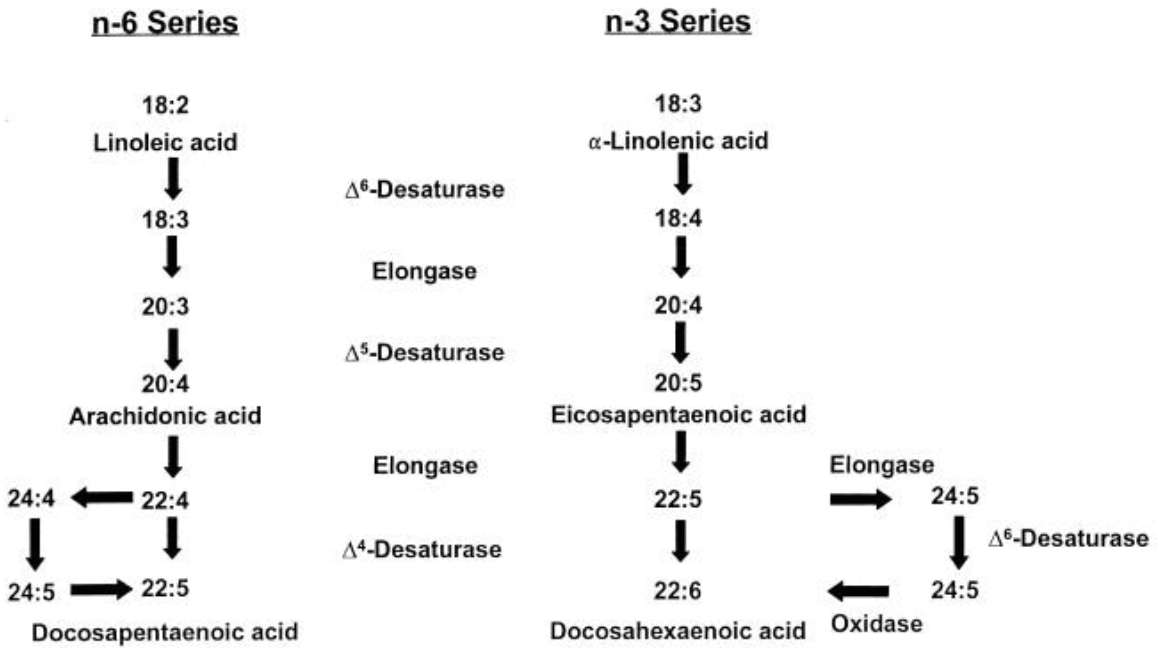


Figura 2 Etapas bioquímicas da síntese dos ácidos graxos poli-insaturados: família ômega-6 e família ômega-3 (LAURITZEN et al., 2001).

O DHA e o AA estão entre os mais importantes constituintes das membranas neuronais. O DHA tem um papel importante no metabolismo encefálico, influenciando o desempenho cognitivo, a acuidade visual, metabolismo de neurotransmissores e desenvolvimento neural (CHALON, 2006; JIMÉNEZ et al., 1997).

Além disso, o EPA e o DHA interagem com outros componentes da membrana celular, especialmente fosfolipídeos. A incorporação desses AGPIs na membranas celulares tem grande impacto nas funções celulares (SONG et al., 2016a).

A presença desses ácidos graxos na membrana fosfolipídica aumenta a fluidez da membrana e melhora sua permeabilidade, sendo que o DHA tem maior importância nesse processo. Se a ingestão de lipídeos através da dieta não é adequada, o DHA será substituído por outros ácidos graxos, tendo grande impacto nas funções cerebrais (CARLSON, 2001; HASHIMOTO; HOSSAIN; SHIDO, 2006; TREEN et al., 1992; WANG et al., 2002).

Adicionalmente, o EPA e o DHA têm função de reduzir processos inflamatórios. Durante a inflamação, o EPA age como inibidor da ciclooxigenase 2 (COX2), além de inibir também a enzima responsável pela formação do AA e consequente formação de eicosanóides, o que leva à uma modulação da via inflamatória. Ambos EPA e AA são precursores dos eicosanoides, sendo que os

eicosanóides derivados do EPA (série anti-inflamatória) antagonizam os efeitos daqueles derivados do AA (série inflamatória) (MCNAMARA, 2016; MCNAMARA; CARLSON, 2006; SONG et al., 2016b).

Estudos epidemiológicos mostram que uma deficiência na ingestão de AGPIs W-3 está associada a maiores índices de depressão e Alzheimer. Além disso, maior razão entre W-6/W-3 foi encontrada no sangue de pacientes com depressão (CONQUER et al., 2000; RIEMER et al., 2010).

Níveis plasmáticos reduzidos de DHA foram encontrados em crianças e adolescentes do sexo feminino diagnosticadas com depressão. Em outro estudo, pacientes com baixos níveis de EPA e alta razão W-6/W-3 apresentaram sintomas depressivos (CONKLIN et al., 2007; TSUCHIMINE et al., 2015).

Trabalhos de nosso grupo investigaram a eficácia antidepressiva da suplementação com óleo de peixe (rico em EPA e DHA) em humanos e em modelos animais. A suplementação com óleo de peixe em pacientes parkinsonianos diagnosticados com depressão levou a uma melhora nos sintomas depressivos, sugerindo que esse tratamento possa ser utilizado como monoterapia, prevenção ou para potencialização do tratamento com antidepressivos convencionais (DA SILVA et al., 2008).

Em diferentes trabalhos de nosso grupo, observamos que o efeito da suplementação com óleo de peixe em ratos promoveu efeito antidepressivo, visto pela maior frequência de natação no teste da natação forçada modificado; efeito este que está relacionado com o aumento de serotonina (5-HT) no hipocampo e a uma sensibilização dos receptores $5HT_{1A}$ nesta mesma estrutura. No entanto, o mecanismo pelo qual a suplementação leva ao aumento de serotonina ainda não foi esclarecido (CARABELLI et al., 2014b; VINES et al., 2012a).

Trabalhos recentes utilizando o modelo de desafio imunológico com LPS mostram que o efeito antidepressivo do óleo de peixe está relacionado com seu efeito anti-inflamatório, diminuindo a expressão da enzima IDO e restaurando os níveis de serotonina (DANG et al., 2017; SHI et al., 2016).

Com o objetivo de investigar se o efeito anti-inflamatório e consequente inibição da IDO são os únicos mecanismos envolvidos no efeito antidepressivo do ômega-3, decidimos utilizar drogas que interagem com esta enzima, em ratos suplementados com óleo de peixe. Ao nosso conhecimento, este é o primeiro estudo

a utilizar o inibidor direto da enzima IDO - 1-MetilTriptofano (1-MT) - e o inibidor indireto Minociclina, em animais suplementados e utilizando o modelo do LPS, a fim de testar a hipótese de potencialização do efeito antidepressivo da suplementação.

Para isso realizamos 2 experimentos, ambos com suplementação com óleo de peixe por 50 dias e injeção de LPS 24 horas antes dos testes. No experimento 1 os animais receberam tratamento com 1-MT e no experimento 2 com Minociclina.

## 2 JUSTIFICATIVAS

A Organização Mundial de Saúde (OMS) afirma que a depressão é a principal causa de incapacitação, conforme medido por anos vividos com invalidez para o trabalho e o quarto principal contribuinte para a carga global de doenças (WHO, 2012).

O tratamento de primeira linha é eficaz em aproximadamente 50% dos casos, o que reforça a busca por alternativas que possam prevenir a depressão ou ainda potencializar o tratamento convencional (CIPRIANI et al., 2009; TRIVEDI et al., 2006a).

Evidências apontam que o aumento do consumo de alimentos ricos em ômega-3 ou a suplementação ajuda a prevenir a depressão e amenizar os sintomas depressivos. O fato de que este composto apresenta efeito anti-inflamatório, somado ao forte componente inflamatório presente na depressão, faz dele uma alternativa promissora para o combate e prevenção dessa doença (CARABELLI et al., 2014a; PUDELL et al., 2014; SHI et al., 2016).

## 3 OBJETIVOS

### 3.1 Objetivo Geral

Investigar a participação da enzima Indolamina-2,3- dioxygenase (IDO) no efeito antidepressivo da suplementação com óleo de peixe.

## 3.2 Objetivos Específicos

1) Verificar a participação da enzima IDO no efeito antidepressivo da suplementação com óleo de peixe no modelo de depressão com desafio imunológico através da injeção de LPS em ratos submetidos ao teste da natação forçada modificado.
2) Verificar a expressão da enzima Indoleamina 2,3- diozigenase (IDO) no hipocampo.
3) Dosar os níveis de serotonina e seu metabólito no hipocampo através da técnica de Cromatografia Líquida de Alta Eficiência (HPLC).
4) Verificar alterações na motricidade e possível comportamento de ansiedade dos animais através do teste do campo aberto.

# 4 MÉTODOS

## 4.1 Animais

Ratos Wistar com aproximadamente 60 dias de vida foram mantidos no biotério da Universidade Federal do Paraná (UFPR) sob o ciclo 12h claro/12h escuro (luzes acendendo às 7:00) em ambiente com temperadtura controlada (21 ± 2 °C), com ração (NuvitalNuvilab CR1- NuvitalNutrientes S/A, Colombo, Paraná, Brasil) e água a vontade. Todos os protocolos experimentais foram aprovados pelo Comitê de Ética da UFPR (# 795).

## 4.2 Drogas

As drogas 1-methil-DL-triptophano (1-MT, Sigma-Aldrich, USA) na dose de 3mg/Kg i.p (DA SILVA DIAS et al., 2015) e Minociclina (Sigma-Aldrich, EUA) na dose de 60mg/Kg i.p (MOLINA-HERNÁNDEZ et al., 2008a), que bloqueam direta e indiretamente a enzima IDO, respectivamente, foram dissolvidos em salina, com ajuste no pH utilizando Hcl e NaOH. A aplicação dessas drogas se deu 23h, 5h e 1h antes dos testes. O LPS (Escherichia coli, O111:B4, Sigma Aldrich, Catálogo # L2630) também foi dissolvido em salina e injetado via intraperitoneal na dose de 250µg/Kg

(BLUTH; DANTZER; KELLEY, 1992; KONSMAN et al., 2008) 24 horas antes da realização dos testes comportamentais.

### 4.3 Testes Comportamentais

#### 4.3.1 Campo Aberto

O campo aberto consiste numa arena circular (1 metro de diâmetro), limitada por parede de 40 cm de altura, iluminada por quatro lâmpadas de 60 W. O piso da arena é forrado de material preto, sem divisões aparentes. Os animais foram colocados individualmente na área central, e permitidos a explorar livremente a arena durante 5 minutos. Durante este período, o software Smart System ® Junior (Panlab, Harvard Apparatus, Espanha) foi utilizado para medir o comportamento de locomoção do animal, através da análise da distância e do tempo gasto em cada área do campo aberto (centro e periferia). Uma solução de água-etanol de 5% foi usada para limpar o campo aberto antes de cada teste para eliminar possível viés devido aos odores deixados pelos ratos anteriores.

#### 4.3.2 Teste da Natação Forçada Modificado

O teste da natação forçada foi efetuado conforme descrito por Cryan, Markou e Lucki (2002) (CRYAN; MARKOU; LUCKI, 2002a). Os animais foram colocados em cilindros plásticos opacos (diâmetro: 20 x altura: 50cm) preenchidos com coluna de água de 30cm, a 24 $\pm$ 1°C. Os animais foram submetidos a uma sessão de treino com 15 minutos de duração, e 25 horas depois do treino passaram pelo teste com duração de 5 minutos cada animal. As sessões de testes foram gravadas por uma câmera digital posicionada sobre o cilindro para posterior análise. Durante a sessão de teste foram mensurados os comportamentos de imobilidade (ratos que permanecem boiando sem se movimentarem), natação (movimentos de natação através do cilindro) e escalada (movimentos para cima com as patas dianteiras sobre as paredes do cilindro). A água do cilindro foi trocada após os testes de cada animal, para evitar ou minimizar possíveis interferências, e após o teste os animais foram e reacomodados em suas respectivas caixas com aquecedor posicionado próximo a estas.

## 4.4 Delineamento Experimental

Foram realizados 2 experimentos independentes. Experimento 1: os animais foram randomicamente distribuídos em 2 grupos: não-suplementados, chamado de grupo controle (C, n=59) e suplementados com óleo de peixe (FO, n=56). Os animais do grupo suplementado receberam suplementação diária de 3g/Kg de óleo de peixe contendo 18% de EPA e 12% de DHA administrado via gavagem. As cápsulas de óleo de peixe foram doadas pelo laboratório Herbarium (Colombo, PR, Brasil). O grupo controle recebeu apenas água no mesmo volume. Ambos os grupos receberam ração padrão, e a composição de ácidos graxos desta foi a mesma previamente demonstrada (FERRAZ et al., 2011). O protocolo de suplementação de 50 dias foi baseado em estudo anterior de nosso grupo (MORI et al., 2017).

Após o término da suplementação, os grupos foram redistribuídos em 4 novos grupos dentro dos 2 grupos formados antes (FO e C): C/SAL/SAL (não-suplementados, receberam apenas injeções de salina, n=16), C/LPS/SAL (não-suplementados,com injeção de LPS e salina, n=16), C/SAL/1MT (não-suplementados, com injeção de salina e 1-MT, n=13) e C/LPS/1-MT (não-suplementados, injeção de LPS e 1-MT, n=14), FO/SAL/SAL (suplementados, receberam apenas injeções de salina, n=14), FO/LPS/SAL (suplementados, receberam LPS e salina, n=14), FO/SAL/1MT (suplementados, com injeções de salina e 1-MT, n=14) e FO/LPS/1MT (suplementados, receberam LPS e 1-MT, n=14).

Experimento 2: o protocolo foi semelhante ao do experimento 1, porém os animais receberam tratamento com minociclina (MINO) ao invés de 1-MT. Os grupos iniciais também foram divididos em não-suplementados (C, n=64) e suplementados (FO, n=64). Após o término da suplementação: C/SAL/SAL (n = 16), C/LPS/SAL (n = 16), C/SAL/MINO (n = 16), C/LPS/MINO (n = 16), FO/SAL/SAL (n = 16), FO/LPS/SAL (n = 16), FO/SAL/MINO (n = 15) e FO/LPS/MINO (n = 17).

1 hora antes da injeção de LPS, os animais foram submetidos ao pré-teste de 15 minutos da natação forçada modificado. A injeção do LPS foi feita 24h antes dos testes comportamentais e o tratamento com 1-MT e Minociclina se deu 23h (1 hora após injeção do LPS), 5h e 1h antes dos testes (DA SILVA DIAS et al., 2015). No dia seguinte, os animais foram submetidos ao teste do campo aberto e à sessão de teste da natação forçada modificado.

Após os testes comportamentais, os ratos foram decapitados e os hipocampos dissecados para investigar o efeito do tratamento com ômega-3 sobre os níveis de serotonina e seu metabólito, 5-HIAA, através da técnica de cromatografia líquida de alta eficiência (HPLC) e verificar a expressão da enzima IDO através da técnica de western blot.

Todos os animais foram pesados antes do pré-teste da natação forçada e no dia seguinte, imediatamente antes da sessão de testes.

### 4.5 Análises Neuroquímicas (HPLC)

Após os testes comportamentais, os animais foram decapitados, os encéfalos removidos, e os hipocampos foram dissecados e congelados em freezer -80°C até a realização do HPLC. Para realização do HPLC, o hipocampo esquerdo de cada animal foi homogeneizado em 500mL de solução de extração contendo 0.1M de ácido perclórico, 0.4Mm de metabissulfito sódico e 0.2mM de EDTA (Merck). Os homogenados foram centrifugados a 20.000 rpm por 10 minutos, filtrados através de membrana de 0.22mm e armazenados em freezer -80 graus para análises posteriores. A realização das etapas seguintes foram feitas de acordo com trabalho anterior (MACHADO; TUFIK; SUCHECKI, 2008).

### 4.6 Western Blot

Após a ortotanásia dos animais, o encéfalo foi dissecado e os hipocampos removidos. Para realização do western blot e investigação da expressão da IDO, o hipocampo direito foi homogeneizado em tampão de lise gelado (25 mM Tris-HCl pH 7,4, 150 mM de NaCl, 5184 mM de MgCl2, 0,3% de Triton X-100 e inibidores de protease(Roche).

A concentração de proteínas foi determinada utilizando o ensaio de Bradford. Os lisados hipocampais (40 ug) foram fervidos em tampão de amostra redutor durante 5 minutos a 95ºC e depois feita a eletroforese para separação das proteínas a 12% SDS-PAGE, seguido por transferência de proteínas para membranas de nitrocelulose (GE Healthcare). As membranas foram bloqueadas com TBS-Tween 20 (120 mM de NaCl, 20 mM de Tris-HCl a pH 7,4 e 0,05% de Tween-20) contendo 5% de leite em

pó desnatado e incubadas com anticorpo anti-IDO (Santa Cruz) na titulação de 1:500. O anticorpo anti-βactina (Sigma) 1:5000 foi usado para detectar a expressão da proteína Beta Actina, usado como normalizador. Em ambos foi utilizado o anticorpo secundário anti-mouse (sigma) 1:4000.

As reações foram desenvolvidas com Westar ECL-Sun (Cyanagen) ou Westar SuperNova (Cyanagen) substrato quimioluminescente para o Western Blot e a exposição foi feita no Sistema de Imagem Amersham$^{TM}$ Imager 600 system (GE Healthcare). As bandas foram quantificadas por análise de densitometria utilizando o software Image J (EUA).

### 4.7 Análise Estatística

As diferenças entre os grupos nos parâmetros de variação do peso, testes comportamentais, concentração de neurotransmissores e expressão da enzima IDO foram analisadas através da análise de variância (ANOVA) de 3 vias, para os fatores: suplementação x LPS x 1-MT/MINO. Em seguida, foi realizado o pós-teste de Tukey. Os dados são representados como média ± EPM. Diferenças foram consideradas estatisticamente significantes quando p≤0,05.

## 5 ARTIGO CIENTÍFICO

# Indoleamine-2,3-dioxygenase inhibition does not seem to be essential for fish oil antidepressant effect

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## Abstract

Depression is a severe psychiatric disease, with a high prevalence during life. Is twice common in women and according World Health Organization approximately 320 million people suffer from depression worldwide.

The Monoaminergic Theory is the basis of current treatment, but there are many evidence pointing out to the role of inflammation on pathogenesis of depression. Depressed patients have an increased rate of autoimmune disorders, and patients with inflammatory diseases, such as cancer, HIV and rheumatoid arthritis have higher rates of depression.

In animal models, systemic injection of bacterial endotoxin lipopolysaccharide evokes an acute inflammatory response and 24h after the injection rodents exhibit a depressive-like behavior, seen by higher immobility in the forced swim test.

The link between inflammation and depression is made by the enzyme Indoleamine-2,3-dioxygenase (IDO), which is activated by pro-inflammatory cytokines. This enzyme catabolizes tryptophan into kynurenines, decreasing the levels of serotonin and promoting depressive symptoms.

Anti-inflammatory compounds, such as Omega-3 Polyunsaturated Fatty Acids, have antidepressant activity, increasing serotonin levels in hippocampus of rats.

Here we hypothesized that this antidepressant effect of omega-3 is due to its anti-inflammatory properties, and the mechanism by which increases serotonin is inhibiting IDO.

In order to test this hypothesis, we performed two independent experiments, using the LPS model in fish-oil supplemented animals. In the first experiment, animals received a pre-treatment with 1-MT and in the experiment 2 they were treated with minocycline. The use of these two inhibitors of IDO was made to test whether there was a potentiation of omega-3 antidepressant activity.

The supplementation was made for 50 days and LPS was injected 24h before the open field test and the modified forced swimming test. The pretreatment with the inhibitors was made 23h, 5h and 1h before these behavioral tests.

After the tests, the hippocampi were obtained for quantification of serotonin and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) by HPLC and IDO expression by western blot.

We found a depressive-like state induced by LPS, which was blocked by 1-MT, Minocycline and fish-oil supplementation. Also, the supplemented animals presented higher swimming behavior compared to 1-MT and Minocycline, and no augmentation of supplementation effect was found. Minocycline and fish oil suppressed IDO expression, with no additional effects in these treatments combined. LPS induced a decrease in serotonin levels and an increase in 5-HIAA/5-HT ratio, both blocked by the anti-inflammatory compounds minocycline and fish oil. The antidepressant-like behavior induced by omega-3 was related to an increase in serotonin levels in hippocampus, compared to all non-supplemented groups. Given the fact that IDO expression is not significant in control animals and the supplemented animals who received only saline also presented higher levels of serotonin highlight the idea that inhibition of this enzyme is not the only explanation for omega-3 antidepressant effect and possibly this compound increases serotonin through another mechanism.

## Introduction

Depression is a common mental disorder, with more than 320 million people affected, and it is the leading cause of disability worldwide. It's more common in women and it is a major contributor to the overall global burden of disease. (WORLD HEALTH ORGANIZATION, 2017).

The pathophysiology of depression it's not fully elucidated but there are many evidences showing a bi-directional relationship between depression and inflammation. Depressed patients have an increased rate of autoimmune disorders, and patients with inflammatory diseases, like cancer, HIV and rheumatoid arthritis have higher rates of depression (EVANS et al., 2005) . Also, increased levels of pro-inflammatory cytokines, such as IL-1B, IL-6 and TNF-α have been found in patients with depression (FELGER; LOTRICH, 2013; PASCO et al., 2010).

Systemic injection of bacterial endotoxin lipopolysaccharide (LPS) provokes an acute inflammatory response and leads to depressive-like behavior in rodents (CHRISTIAN R. H. RAETZ AND CHRIS WHITFIELD., 2008; DANTZER et al., 2008).

Pro-inflammatory cytokines have been shown to reduce serotonin (5-HT) levels by induction of the enzyme indoleamine - 2,3 – dioxygenase (IDO), which catabolizes tryptophan, an essential precursor of 5-HT, into kynurenines, leading to depressive state (DANTZER et al., 2011a; MAES et al., 2011a)

These effects can be reversed by treatment with antidepressants, for example the Selective Serotonin Reuptake Inhibitors (SSRIs), and with other compounds that have antidepressant effects, such as Omega-3 Polyunsaturated Fatty Acids (W-3 PUFAs) (GALECKI; MOSSAKOWSKA-WÓJCIK; TALAROWSKA, 2017; SHI et al., 2016).

It is known that W-3 PUFAs are critical components of biological membranes and play key roles in cell integrity, homeostasis, development and function (BAZAN; MOLINA; GORDON, 2012). Some studies show that a deficiency of omega-3 are present psychiatric diseases, especially depression (MCNAMARA, 2013). Fish-oil supplementation (rich in W-3 PUFAs) during pregnancy and lactation produces an antidepressant effect in animal models, related to an increase in 5-HT levels (CARABELLI et al., 2014b; PUDELL et al., 2014; VINES et al., 2012a). Interestingly, omega-3 also presents anti-inflammatory effects acting through the regulation of

inflammatory gene expression, especially cytokines and chemokines and the decrease of inflammatory prostaglandins and eicosanoids (CALDER, 2013).

Given all these evidence pointing out to the involvement of the IDO pathway and inflammation, we hypothesized that the W-3 PUFAs's antidepressant activity is due to its anti-inflammatory effect. In order to test this hypothesis, we performed two experiments, both with chronic fish oil supplementation and an acute immune challenge with LPS. In the experiment 1, the animals received a pre-treatment with the IDO competitive inhibitor 1-metil-tryptophan and in the experiment 2, with the indirect inhibitor Minocycline.

## Methods

### *Animals*

60 days-old male Wistar rats were kept under a 12 h light /12 h dark cycle (lights on at 07:00 am) in a controlled temperature room (21 ± 2 °C), with food (rat chow, NuvitalNuvilab CR1- NuvitalNutrientes S/A, Colombo, Paraná, Brazil) and water *ad libitum*. All experiments were approved by the Animal Experimentation Ethics Committee of the Universidade Federal do Paraná (# 795) and were performed according to the Guide for the Care and Use of Experimental Animals (Canadian Council on Animal Care).

### *Drugs*

1-methyl-DL-tryptophan (1-MT, Sigma-Aldrich, USA) at dose of 3 mg/kg, i.p (DA SILVA DIAS et al., 2015) and minocycline (Sigma-Aldrich, USA) at dose of 60 mg/kg i.p.(MOLINA-HERNÁNDEZ et al., 2008a), which directly or indirectly blocks IDO, respectively, were dissolved in saline with several drops of diluted HCl and NaOH for pH adjustment.

LPS (Escherichia coli, O111:B4, Sigma Aldrich, Catalogue # L2630) was dissolved in saline and administered intraperitoneally in a dose of 250ug/Kg (BLUTH; DANTZER; KELLEY, 1992; KONSMAN et al., 2008) 24h before drug application and behavioral tests.

*Experimental Design*

Two independent experiments were performed. Experiment 1: animals were randomly distributed into 2 experimental groups: non-supplemented, also nominated Control (C, n=59) and supplemented with fish oil (FO, n=56). The animals in the FO group received a daily supplementation of 3.0g/kg of fish oil containing 18% of EPA and 12% of DHA (kindly donated by Laboratório Herbarium Botânico S/A, Colombo, Paraná, Brazil), administered by gavage. The control group received only water at the same volume. Both groups were fed with regular chow, and its fatty acid composition was the same as the reported previously (FERRAZ et al., 2011). The FO group was supplemented for 50 days, based on previous work from our group (MORI et al., 2017). After the supplementation, groups were allocated in 4 other groups inside the FO and C groups: C/SAL/SAL (non-supplemented, received only saline injections, n=16), C/LPS/SAL (non-supplemented, with LPS and saline injections, n=16), C/SAL/1MT (non-supplemented, with saline and 1-MT injections, n=13), C/LPS/1MT (non-supplemented, LPS and 1-MT injections, n=14), FO/SAL/SAL (supplemented, saline injections only, n=14), FO/LPS/SAL (supplemented, with LPS and saline injections, n=14), FO/SAL/1MT (supplemented, with saline and 1-MT injections, n=14) and FO/LPS/1-MT (supplemented, with LPS and 1-MT injections, n=14).

Experiment 2: the protocol was similar, but the animals were treated with Minocycline (MINO), instead of 1-MT. The groups were: non-supplemented (C, n = 64) and supplemented (FO, n = 64). After 50 days of supplementation: C/SAL/SAL (n = 16), C/LPS/SAL (n = 16), C/SAL/MINO (n = 16), C/LPS/MINO (n = 16), FO/SAL/SAL (n = 16), FO/LPS/SAL (n = 16), FO/SAL/MINO (n = 15) and FO/LPS/MINO (n = 17).

The pre-test session of Modified Forced Swim Test (MFST) was performed 1 hour before LPS injection. 24h after LPS injection all groups were assessed for locomotor and depressive-like behaviors by the Open Field test and MFST, respectively, and the treatment with 1-MT or Minocycline was performed 23h, 5h and 1h before tests. The protocol of treatment with these two drugs was based in a previous study from our group (DA SILVA DIAS et al., 2015). After behavioral tests, rats were decapitated, and the hippocampi were dissected to investigate long-term effects of ω-3 PUFA on monoamines content and their metabolites by high-performance liquid

chromatography (HPLC). Also, the expression of indoleamine-2,3-dioxygenase (IDO) in this brain area was evaluated by Western Blot.

All animals were weighted 1 hour before LPS injection and immediately before the tests.

*Open Field Test*

The open-field (OF) test was performed in a circular arena (1 m diameter) limited by a 40 cm-high wall and illuminated by four 60 W lamps (Broadhurst, 1960). The arena's floor was black, with no apparent divisions. The subjects were individually placed in the central area, and allowed to freely explore the arena for 5 minutes. During this period, the Smart® Junior System (Panlab, Harvard Apparatus, Spain) was used to measure the subject's locomotion behavior, by analyzing distance, and time spent in each area of the OF (central and periphery). The open-field was cleaned with a 10% water-ethanol solution before each behavioral testing to eliminate possible bias due to odors left by previous rats.

*Modified Forced Swim Test*

This is a modified version of the Porsolt test and was carried out as previously described (CRYAN; MARKOU; LUCKI, 2002b). Briefly, rats were placed, individually, in an opaque plastic cylinder (diameter 20 cm; height 50 cm) containing water up to 30 cm (24 ±1ºC); on day 1 the rats remained in the cylinder for 15 min (training session) and 24 h later they were placed back and tested for 5 min (test session). The test session was video recorded via a camera positioned above the cylinder for subsequent analysis. The behaviors assessed during the test session were: immobility (when the rat stopped all active behaviors and remained floating in the water with minimal movements, with its head just above the water), swimming (movements throughout the swim cylinder) and climbing (upward directed movements of the forepaws along the cylinder walls). During the 5-min session, the predominant behavior within each 5-s interval was recorded. The water was changed and the cylinder rinsed with clean water after each session. After the training and the test sessions, the animals were dried and placed in their home cages.

*Western Blot*

The hippocampi were homogenized in ice-cold lysis buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM MgCl2, 0.3 % Triton X-100, and Complete Protease Inhibitor Cocktail (Roche)) and the protein concentration determined using the Bradford assay. Hippocampal lysates (40µg) were boiled in Laemmli sample buffer for 5 min at 95 °C for 5 min and then subjected to 12%SDS-PAGEunder reducing condition followed by transference of proteins to nitrocellulose membranes (GE Healthcare). Membranes were blocked with TBS-Tween 20 (120 mM NaCl, 20 mM Tris–HCl pH 7.4, and 0.05 % Tween- 20) containing 5 % non-fat dry milk and analyzed with anti-IDO antibody (Santa Cruz). Anti-βactin antibody (Sigma) was used for protein loading control. Reactions were developed with Westar ECL-Sun (Cyanagen) or Westar SuperNova (Cyanagen) chemiluminescent substrate for Western blot and exposed to Amersham$^{TM}$ Imager 600 system (GE Healthcare). The bands were quantified by densitometry analysis using ImageJ software (USA).

*Neurochemical quantification (HPLC)*

The animals were decapitated and their brains were removed and the hippocampi were dissected on a cold surface. The tissue samples were weighed individually and homogenized by sonication in 500 mL of extraction solution (0.1 M perchloric acid containing 0.4 mM sodium metabissulfite and 0.2 mMethylenediaminetetraacetic acid). The homogenates were centrifuged at 20,000 x *g* for 10 min, then filtered through 0.22mm membrane and stored at -80°C for further analysis. Precipitates were dissolved in 0.1 N NaOH and assayed for protein estimation (Bicinchoninic acid method, Pierce Chemical, Rockford, IL). Supernatants were submitted to fast isocratic separation through a C18 HPLC reversed-phase column system (Spheri-5, C18, ODS, 5 mm, 25 cm, 4.6 mm column; linked to a New-Guard Cartridge Column, RP-18,7 mm pre-column; Perkin Elmer Brownlee Columns, Shelton, CT) and electrochemically detected using an amperometric detector (L-ECD-6A, Shimadzu, Japan), by oxidation on glass carbon electrode at +850 mV in relation to an Ag-AgCl reference electrode (MACHADO; TUFIK; SUCHECKI, 2008). The mobile phase consisted of 0.163 M citric acid, 0.06 M sodium phosphate dibasic

anhydrous, 0.69 mMoctyl sodium sulfate, 12 mMethylenediaminetetraacetic acid, acetonitrile 4%, tetrahydrofuran 1.7% and orthophosphoric acid sufficient to bring the pH to 2.85, diluted in double distilled water. The mobile phase was filtered through a 0.2 mm filter membrane, degassed under vacuum and delivered at a flow rate of 1.2 mL/min (HITACHI Pump System L-7100). Each sample was analyzed in duplicate for concentrations of serotonin (5-HT) and its non-conjugated metabolite 5-hydroxyindoleacetic acid (5-HIAA). The recovery of the analytes was determined by adding a fixed concentration of internal standard DHBA (dihydroxybenzylamine) before tissue homogenization. An automatic injector (HITACHI L-7250, cut injection method) was utilized to improve the reproducibility of injections. All standards and salts were purchased from Sigma (USA) and the solvents (HPLC grade) were purchased from J.T. Baker (USA).

*Statistical analysis*

Differences among groups in body weight changes, behavioral tests and biochemical and molecular analysis were examined by three-way analysis of variance (ANOVA) – supplementation, LPS, 1-MT (experiment 1) or Minocycline (experiment 2) - followed by Tukey's *post hoc* test. The results are reported as mean ± S.E.M. Differences were considered statistically significant when $p \leq 0.05$.

## Results

### *Experiment 1*

*Body Weight (BW)*

For BW change, three-way ANOVA revealed an effect FO [$F(1,107) = 21.8$; $p \leq 0.00009$], LPS [$F(1,107) = 138.94$; $p \leq 0.000001$] and an interaction between FO and LPS [$F(1,107) = 25.6$; $p \leq 0.000002$]. Tukey's post hoc test showed that LPS provoked a pronounced weight loss (C/LPS/SAL and C/LPS/1MT vs all other groups; $p \leq 0.0002$). The groups whom received 2 months of fish-oil supplementation and were injected with LPS (FO/LPS/SAL and FO/LPS/1MT) exhibited a slightly weight loss compared to the

non-supplemented groups (p≤0.0003). There was no effect of 1-MT [F(1,107) = 0.16; n.s.] and no interaction between: FO and 1-MT [F(1,107) = 0.3; n.s.], LPS and 1-MT [F(1,107) = 0.01; n.s.] or between FO, LPS and 1-MT [F(1,107) = 0.04; n.s.] (Fig 1).

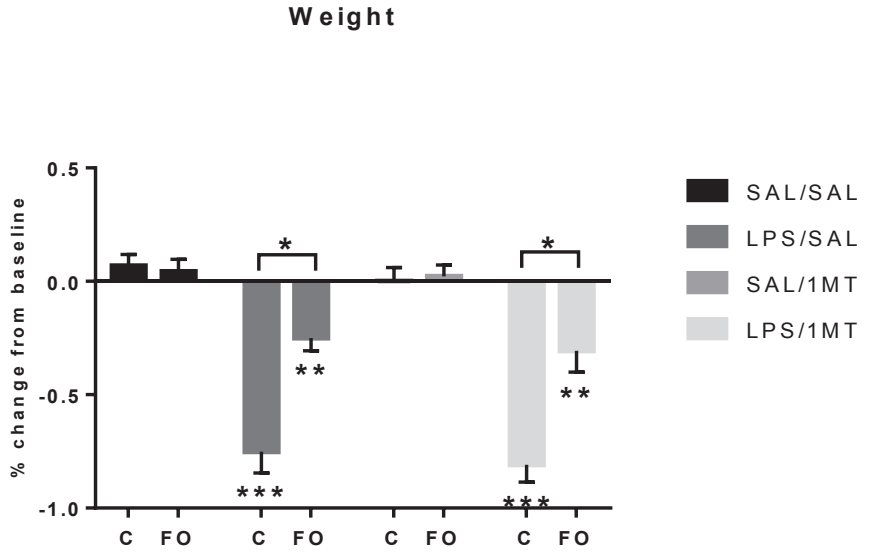


Fig 1. 24h body weight (% change from baseline). C: non-supplemented group (control); FO: Supplemented group (FIsh Oil). C/SAL/SAL (control group that received only saline injections, n=16); C/LPS/SAL (control group, with Lipopolysacharide and saline injections, n=16); C/SAL/1MT (control group, with injection of 1-MT and saline, n=13); C/LPS/1MT (control group, with LPS and 1-MT injections, n=14). FO/SAL/SAL (supplemented, with saline injections, n=14); FO/LPS/SAL, (supplemented, with Lipopolysacharide and saline injections, n=14); FO/SAL/1MT (supplemented, with saline and 1-MT injections, n=14); FO/LPS/1MT (supplemented, with LPS and 1-MT injections, n=14). Three-way ANOVA followed by Tukey's post-hoc test. Values are expressed as mean± S.E.M. ***p≤0.0002 compared to all groups; **p≤0.05 compared to control and supplemented SAL/SAL and SAL/1MT groups; *p≤0.0003

*Open Field Test*

Figure 2 shows total distance (A), central distance (B), peripheral distance (C), time in center (D) and time in periphery (E) in the open field test. Three-way ANOVA did not show any difference for supplementation in total distance [F(1,107)=0.0102; n.s], central distance [F(1,107)=0.04; n.s], peripheral distance [F(1,107)=0.37; n.s], time in center [F(1,107)=0.28; n.s] and time in periphery [F(1,107)=0.28; n.s]. No difference for LPS: in total distance [F(1,107)=0.08; n.s], central distance [F(1,107)=0.29; n.s], peripheral distance [F(1,107)=0.02; n.s], time in center [F(1,107)=1.69; n.s], and time in periphery [F(1,107)=1.69; n.s], no effect of 1-MT

treatment: total distance [F(1,107)=0.004; n.s], central distance [F(1,107)=0.04; n.s], peripheral distance [F(1,107)=0.27; n.s], time in center [F(1,107)=0.36; n.s], and time in periphery [F(1,107)=0.36; n.s].

Also, there was no interaction between: FO and 1-MT in total distance [F(1,107)=0.67; n.s], central distance [F(1,107)=0.16; n.s], peripheral distance [F(1,107)=0.14; n.s], time in center [F(1,107)=0.16; n.s] and time in periphery [F(1,111)=0.16; n.s]; FO and LPS in total distance [F(1,107)=0.005; n.s], central distance [F(1,107)=0.03; n.s], peripheral distance [F(1,107)=0.18; n.s], time in center [F(1,107)=0.4; n.s] and time in periphery [F(1,107)=0.4; n.s]; 1-MT and LPS in total distance [F(1,107)=0.028; n.s], central distance [F(1,107)=0.13; n.s], peripheral distance [F(1,107)=0.008; n.s], time in center [F(1,107)=0.09; n.s] and time in periphery [F(1,107)=0.09; n.s] and no interaction between FO, 1-MT and LPS for all parameters: total distance [F(1,107)=0.07; n.s], central distance F(1,107)=0.07; n.s], peripheral distance [F(1,107)=0.03; n.s], time in center [F(1,107)=0.00; n.s] and time in periphery [F(1,107)=0.00; n.s].

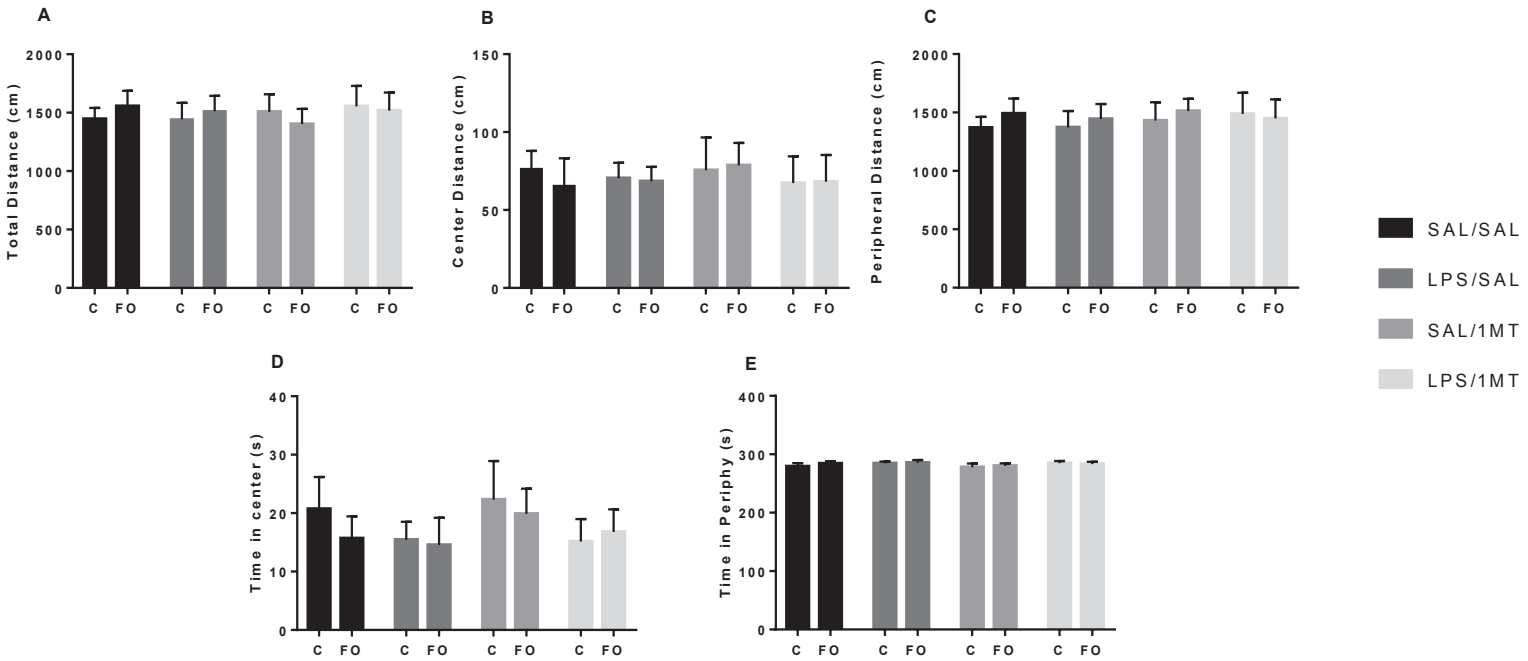


Fig 2 Open Field test. **A** Total Distance **B** Central distance **C** Peripheral distance **D** Time in center **E** Time in periphery. C: non-supplemented group (control); FO: Supplemented group (FIsh Oil). C/SAL/SAL (control group that received only saline injections, n=16); C/LPS/SAL (control group, with Lipopolysacharide and saline injections, n=16); C/SAL/1MT (control group, with injection of 1-MT and saline, n=13); C/LPS/1MT (control group, with LPS and 1-MT injections, n=14). FO/SAL/SAL (supplemented, with saline injections, n=14); FO/LPS/SAL, (supplemented, with Lipopolysacharide and saline injections, n=14); FO/SAL/1MT (supplemented, with saline and 1-MT injections, n=14); FO/LPS/1MT (supplemented, with LPS and 1-MT injections, n=14). Three-way ANOVA. Values are expressed as mean± S.E.M.

Modified Forced Swimming Test

In this test, we analyzed three parameters: immobility, swimming, and climbing frequencies (Fig. 3). Regarding swimming (Fig. 4A), there was an effect of the fish-oil supplementation [$F(1,107)= 106.7$; $p\leq0.0001$], an effect of LPS [$F(1,107)=4.6$; $p\leq 0.04$], an effect of 1-MT [$F(1,107)= 4.92$; $p\leq0.03$], and an interaction between: Fish oil and LPS [$F(1,107)= 8.34$; $p\leq0.005$] and LPS and 1MT [$F(1,107)= 6.82$; $p\leq0.01$]. *Post hoc* test showed that all supplemented groups (FO) displayed higher swimming frequency compared to the all non-supplemented (C) ($p\leq0.02$). The non-supplemented group that received LPS and saline injections (C/LPS/SAL) displayed less frequency of swimming than the other non-supplemented and all FO-groups ($p\leq0.03$). There was no interaction between fish oil and 1MT [$F(1,107)= 1.1$; n.s.] or between fish oil, LPS and 1MT [$F(1,107)= 0.77$; n.s.]

For immobility (Fig. 4B), there was an effect of supplementation [$F(1,107)=107.34$; $p\leq0.0001$], an effect of LPS [$F(1,107)=4.6$; $p\leq0.04$], an effect of 1MT [$F(1,107)=5.8$; $p\leq0.02$]. Also, there was an interaction between fish oil and LPS [$F(1,107)=6.99$; $p\leq0.009$] and between LPS and 1MT [$F(1,107)=5.4$; $p\leq0.02$]. Post hoc test revealed that the non-supplemented group that received only LPS and saline injections (C/LPS/SAL) displayed higher frequency of immobility compared to the other control groups and all FO groups ($p\leq0.03$).

1-MT was effective in prevent the LPS effect, because the immobility frequency presented by the C/LPS/1MT group was similar to the control groups that received only saline (C/SAL/SAL) and 1MT/saline injections (C/SAL/1MT). Also, all the supplemented groups displayed less immobility compared not only to C/LPS/SAL group but to all non-supplemented groups ($p\leq0.02$). There was no interaction between fish oil and 1-MT [$F(1,107)=0.91$; n.s.] or between the three factor: fish oil, LPS and 1-MT [$F(1,107)=0.6$; n.s.].

For climbing behavior (3C) there were no effect of supplementation [$F(1,107)=0.6$; n.s.], LPS [$F(1,107)=0.16$; n.s.], 1-MT [$F(1,107)=0.00009$; n.s.] or any interaction between: fish oil and LPS [$F(1,107)=0.83$; n.s.], fish oil and 1-MT [$F(1,107)=0.022$; n.s.], LPS and 1MT [$F(1,107)=0.23$; n.s.] and fish oil, LPS and 1-MT [$F(1,107)=0.23$; n.s.].

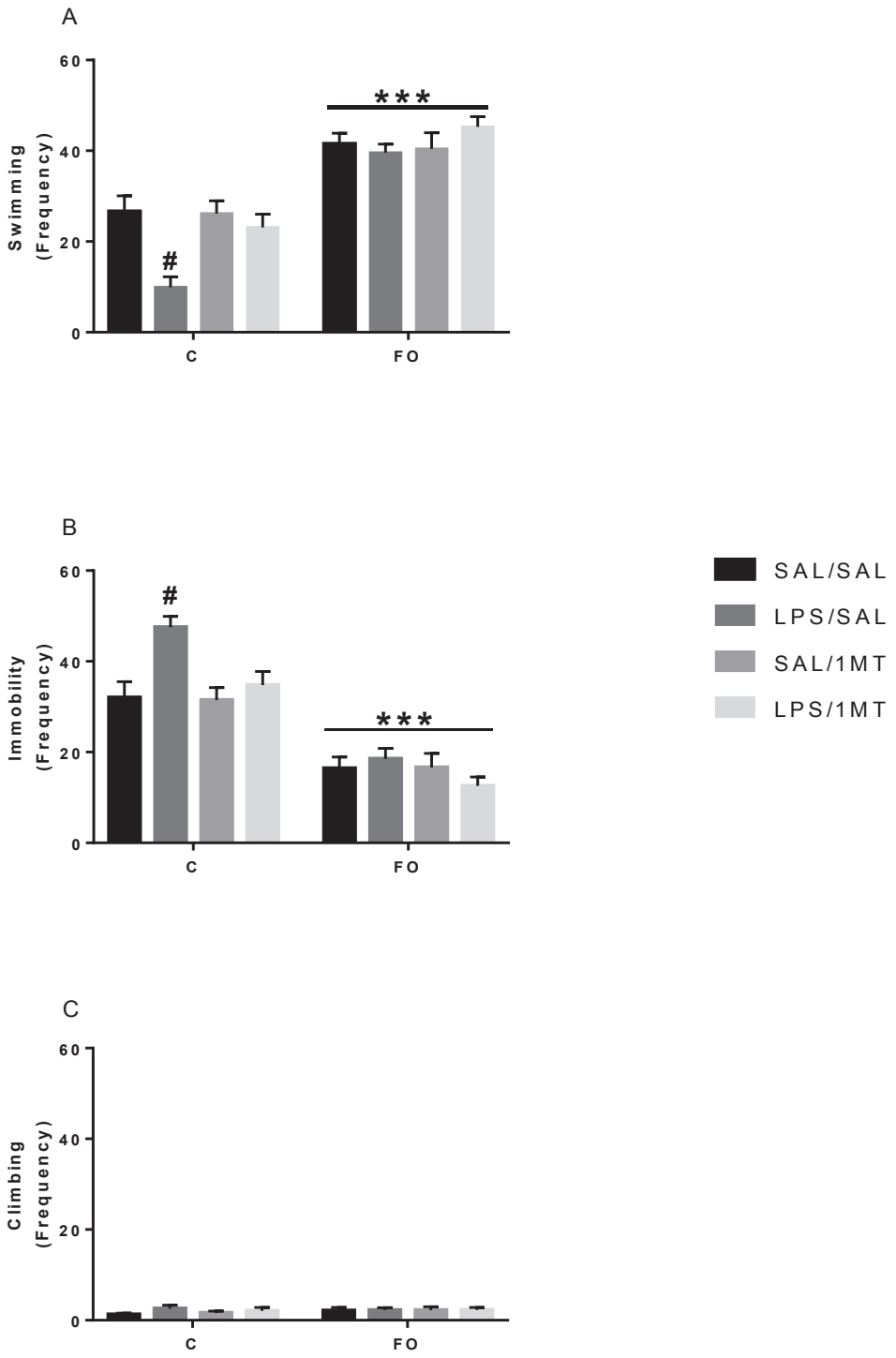


Fig. 3. Modified Forced Swim test. A Swimming; B Immobility; C Climbing. C: non-suplemented (control) groups FO: supplemented groups. C/SAL/SAL (control group who received only saline injections, n=16); C/LPS/SAL (control group, with Lipopolysacharide and saline injections, n=16); C/SAL/1MT(control group, with injection of 1-MT and saline, n=13); C/LPS/1MT (control group, with LPS and 1-MT injections, n=14). FO/SAL/SAL (supplemented, with saline injections, n=14); FO/LPS/SAL(supplemented, with Lipopolysacharide and saline injections, n=14); FO/SAL/1MT ( supplemented, with saline and 1-MT injections, n=14); FO/LPS/1MT (supplemented, with LPS and 1-MT injections). Three-way ANOVA followed by Tukey's post-hoc test. Values are expressed as mean± S.E.M. ***$p \leq 0.02$ compared to all non-supplemented groups; #$p \leq 0.03$ compared to all FO-groups and to C/SAL/SAL, C/SAL/1MT and C/LPS/1MT groups.

*Neurochemical Data (HPLC)*

Figure 4 shows neurochemical quantification in the hippocampus of adult animals (5-HT is shown in Fig. 4a; 5-HIAAin Fig. 4b and the ratio 5-HIAA/5-HT, in Fig. 4c). Regarding 5-HT levels three-way ANOVA revealed an effect of fish oil supplementation [$F(1,37)=116.7$; $p\leq0.00001$], LPS [$F(1,37)=12.98$; $p\leq0.0009$] and an interaction between FO and LPS [$F(1,37)=11.43$; $p\leq0.002$]. Tukey's *post hoc* test showed that the two non-supplemented groups that received LPS (C/LPS/SAL and C/LPS/1MT) exhibited lower levels of serotonin, when compared to the other non-supplemented and all supplemented groups ($p\leq0.05$). The supplemented groups showed higher levels of 5-HT compared to all Control groups ($p\leq0.05$), even when LPS was administered, confirming the protective effect of omega-3. There was no effect of 1-MT on 5-HT levels [$F(1,37)=0.04$; n.s.] or any interaction between FO and 1MT [$F(1,37)=0.06$; n.s.], 1-MT and LPS [$F(1,37)=0.002$; n.s.] or between FO, LPS and 1-MT [$F(1,37)=0.02$; n.s.].

Three-way ANOVA didn't show any differences in 5HIAA levels for FO [$F(1,37)=0.03$; n.s.], 1-MT [$F(1,37)=0.07$; n.s.], LPS [$F(1,37)=0.31$; n.s.].

Also, there was no interactions between the following factors: FO and 1MT [$F(1,37)=0.02$; n.s.], FO and LPS [$F(1,37)=0.08$; n.s.], 1MT and LPS [$F(1,37)=0.022$; n.s.] or FO, LPS and 1MT [$F(1,37)=1.13$; n.s.].

Regarding the ratio 5-HIAA/5-HT three-way ANOVA showed an effect of fish oil [$F(1,37)= 31.33$; $p\leq0.00001$], LPS [$F(1,37)=13.18$ $p\leq0.0008$] and an interaction between FO and LPS [$F(1,37)=13.24$; $p\leq0.0008$]. Post hoc test showed that LPS increased the turnover (C/LPS/SAL and C/LPS/1MT groups compared to the other non-supplemented groups and all FO groups ($p\leq0.05$). No effect was found for treatment with 1-MT [$F(1,37)=0.03$; n.s.] or interaction between FO and 1-MT [$F(1,37)=0.007$; n.s.], 1-MT and LPS [$F(1,37)=0,06$; n.s.] or between FO, LPS and 1-MT [$F(1,37)=0.24$; n.s.].

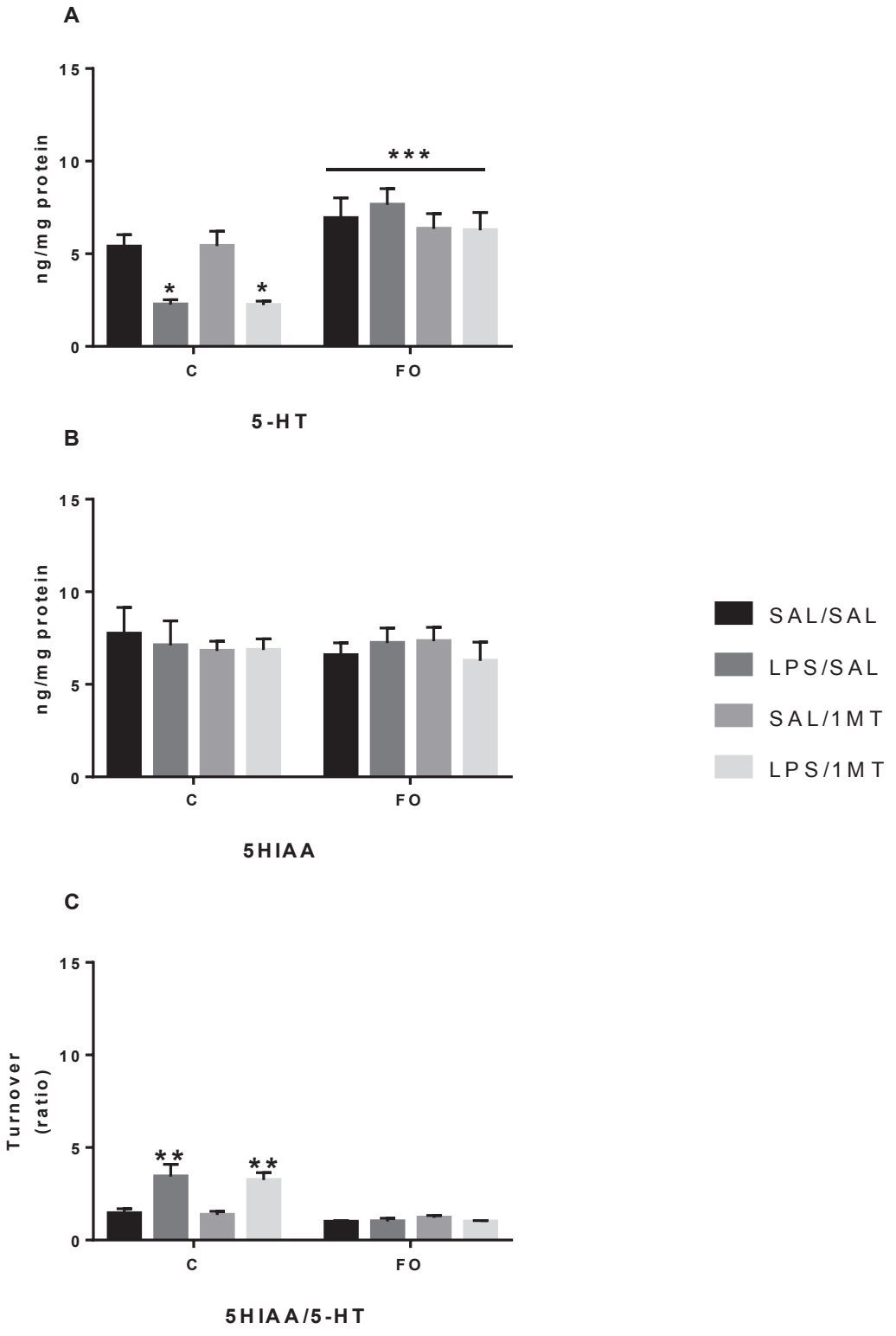


Fig 4. Hippocampal neurochemical data. C: non-supplemented group (control); FO: Supplemented group (FIsh Oil). C/SAL/SAL (control group that received only saline injections, n=6); C/LPS/SAL (control group, with Lipopolysacharide and saline injections, n=8); C/SAL/1MT (control group, with injection of 1-MT and saline, n=5); C/LPS/1MT (control group, with LPS and 1-MT injections, n=6). FO/SAL/SAL (supplemented, with saline injections, n=5); FO/LPS/SAL, (supplemented, with Lipopolysacharide and saline injections, n=5); FO/SAL/1MT (supplemented, with saline and 1-MT injections, n=5); FO/LPS/1MT (supplemented, with LPS and 1-MT injections, n=5). Three-way ANOVA followed by Tukey's post-hoc test. Values are expressed as mean± S.E.M. *p≤0.05 compared to the other non-supplemented and all supplemented groups; ***p≤0.05 compared to the non-supplemented groups **p≤0.05 compared to all groups.

*Western Blot*

Figure 5 shows the expression of IDO in the hippocampus of adult animals. Three-way ANOVA shows an effect of FO [F(1,28)= 7.55; p≤0.01], an effect of LPS [F(1,28)= 6.71; p≤0.02] and an interaction between FO and LPS [F(1,28)= 7.24; p≤0.01]. Tukey's post hoc test showed that LPS increased IDO expression (C/LPS/SAL, C/LPS/1MT vs C/SAL/SAL, S/SAL/1MT, p≤0.05) and fish oil prevented this effect (p≤0.05).

There was no effect of 1-MT [F(1,28)= 0.08;n.s.] and no interaction between: FO and 1-MT [F(1,28)= 0.21;n.s.], LPS and 1-MT [F(1,28)= 0.3; n.s.] or between FO, LPS and 1-MT [F(1,28)= 0.0004; n.s.].

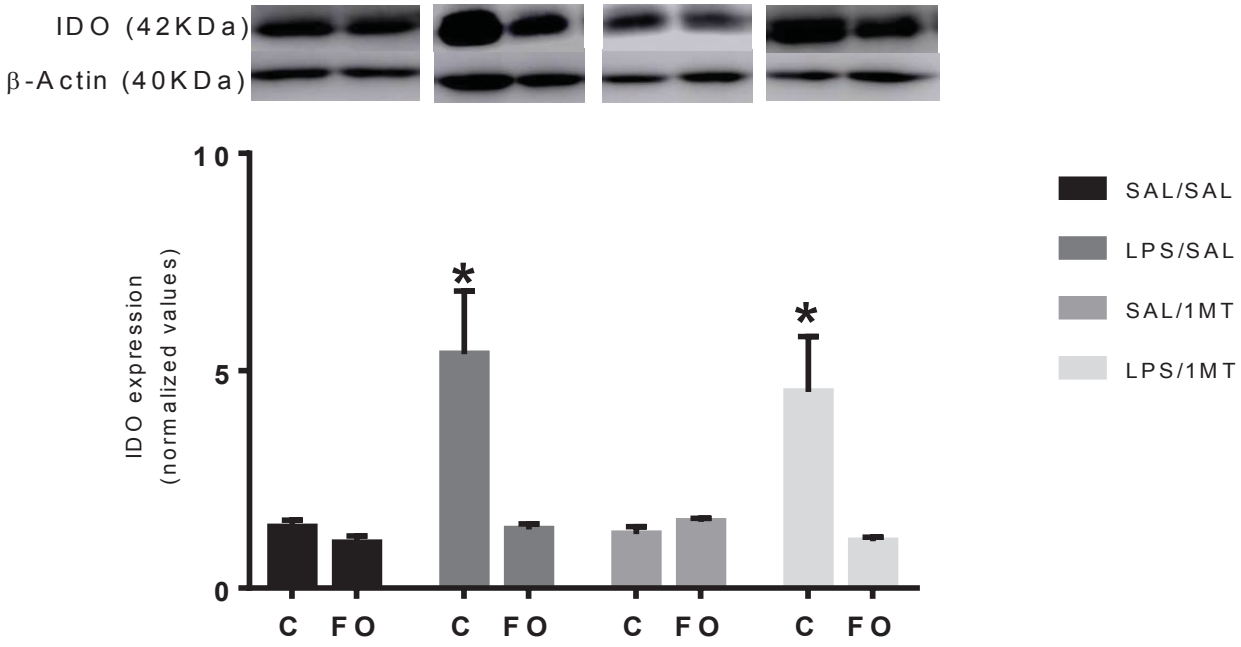


Fig 5 Western Blot analyses: IDO expression (normalized values). C: non-suplemented (control) groups FO: supplemented (fish oil) groups. C/SAL/SAL (control group that received only saline injections, n=6); C/LPS/SAL (control group, with Lipopolysacharide and saline injections, n=4); C/SAL/1-MT (control group, with injection of 1-MT and saline, n=4); C/LPS/1-MT (control group, with LPS and 1-MT injections, n=4). Fish Oil/saline/saline (FO/SAL/SAL, supplemented, with saline injections, n=4); FO/LPS/saline (supplemented, with Lipopolysacharide and saline injections, n=5); FO/SAL/1-MT (supplemented, with saline and 1-MT injections, n=5); FO/LPS/1-MT(supplemented, with LPS and 1-MT injections). Three-way ANOVA followed by Tukey's post-hoc test. Values are expressed as mean± S.E.M. *P≤0.05

***Experiment 2***

*Body Weight*

Three-way ANOVA revealed an effect of supplementation [F(1,120) = 5.7; p≤0.02], LPS [F(1,120) = 58.4; p≤0.000001], MINO [F(1,120) = 26.5; p≤0.0001] and an interaction between FO and LPS [F(1,120) = 9.02; p≤0.003] and between LPS and MINO [F(1,120) = 26.8; p≤0.0001]. *Post hoc* test showed that C/LPS/SAL exhibited a body weight decrease compared to all groups (p≤0.0001) and those treated with Minocycline maintained their weight. The supplemented-LPS group exhibited a slight BW decrease compared to control-LPS group (p≤0.0002).

There was no interaction between FO and MINO [F(1,120) = 1.2; n.s.] and FO, LPS and MINO [F(1,120) = 3.13; n.s.] (Fig 6).

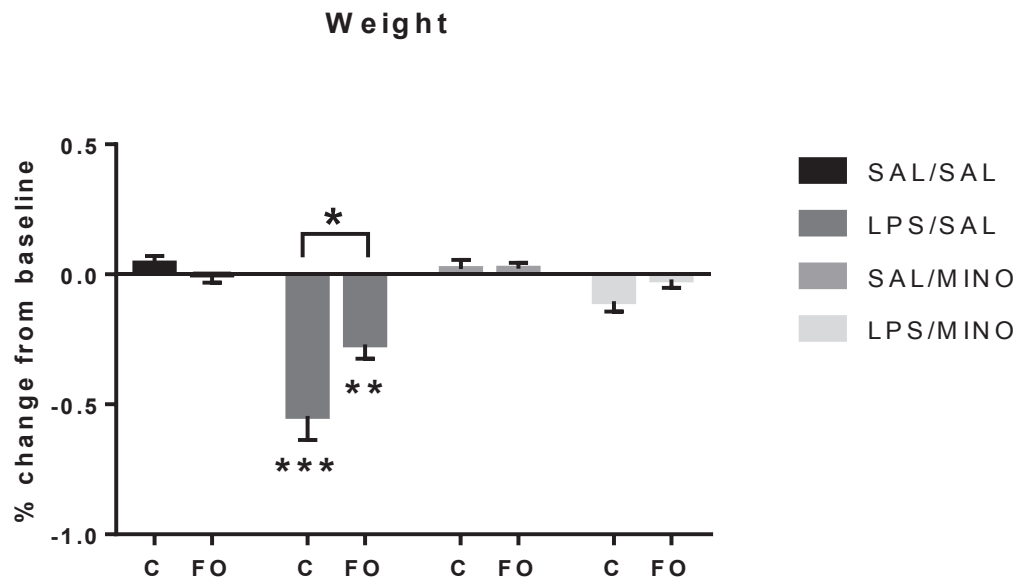


Fig 6. 24h Body weight change (% from baseline). C: non-supplemented group (control); FO: Supplemented group (FIsh Oil). C/SAL/SAL (control group that received only saline injections, n=16); C/LPS/SAL (control group, with Lipopolysacharide and saline injections, n=16); C/SAL/MINO (control group, with injection of minocycline and saline, n=16); C/LPS/MINO (control group, with LPS and Minocycline injections, n=16). FO/SAL/SAL (supplemented, with saline injections, n=16); FO/LPS/SAL (supplemented, with Lipopolysacharide and saline injections, n=16); FO/SAL/MINO (supplemented, with saline and minocycline injections, n=15); FO/LPS/MINO (supplemented, with LPS and Minocycline injections). Three-way ANOVA followed by Tukey's post-hoc test. Values are expressed as mean± S.E.M. ***p≤0.0001 compared to all groups; **p≤0.01 compared to SAL/SAL, SAL/MINO and LPS/MINO groups; *p≤0.0002

*Open Field Test*

Figure 7 shows total distance (A), central distance (B), peripheral distance (C), time in center (D) and time in periphery (E) in the open field test. Three-way ANOVA did not show any difference for parameters: for supplementation in total distance [F (1,120) =0.09; n.s], central distance [F(1,120)=0.05; n.s], peripheral distance [F(1,120)=0.004; n.s], time in center [F(1,120)=1.94; n.s] and time in periphery [F(1,120)=1.94; n.s]. No effect of LPS: in total distance [F(1,120)=0.003; n.s], central distance [F(1,120)=0.004; n.s], peripheral distance [F(1,120)=0.04; n.s], time in center [F(1,120)=0.84; n.s], and time in periphery [F(1,120)=0.84; n.s]; no effect of MINO: total distance [F(1,120)=0.0079; n.s], central distance [F(1,120)=0.05; n.s], peripheral distance [F(1,120)=0.11; n.s], time in center [F(1,120)=0.47; n.s], and time in periphery [F(1,120)=0.47; n.s].There wasn't any interaction between: FO and MINO in total distance [F(1,120)=0.0001; n.s], central distance [F(1,120)=0.51; n.s], peripheral distance [F(1,120)=0.14; n.s], time in center [F(1,120)=0.01; n.s] and time in periphery [F(1,120)=0.01; n.s]; FO and LPS in total distance [F(1,120)=0.12; n.s], central distance [F(1,120)=0.29; n.s], peripheral distance [F(1,120)=0.01; n.s], time in center [F(1,120)=0.15; n.s] and time in periphery [F(1,120)=0.15; n.s]; MINO and LPS in total distance [F(1,120)=0.08; n.s], central distance [F(1,120)=0.34; n.s], peripheral distance [F(1,120)=0.008; n.s], time in center [F(1,120)=0.0028; n.s] and time in periphery [F(1,120)=0.00; n.s] and no interaction between FO, MINO and LPS for all parameters: total distance [F(1,120)=0.02; n.s], central distance [F(1,120)=0.6; n.s], peripheral distance [F(1,120)=0.0005; n.s], time in center [F(1,120)=0.20; n.s] and time in periphery [F(1,120)=0.20; n.s].

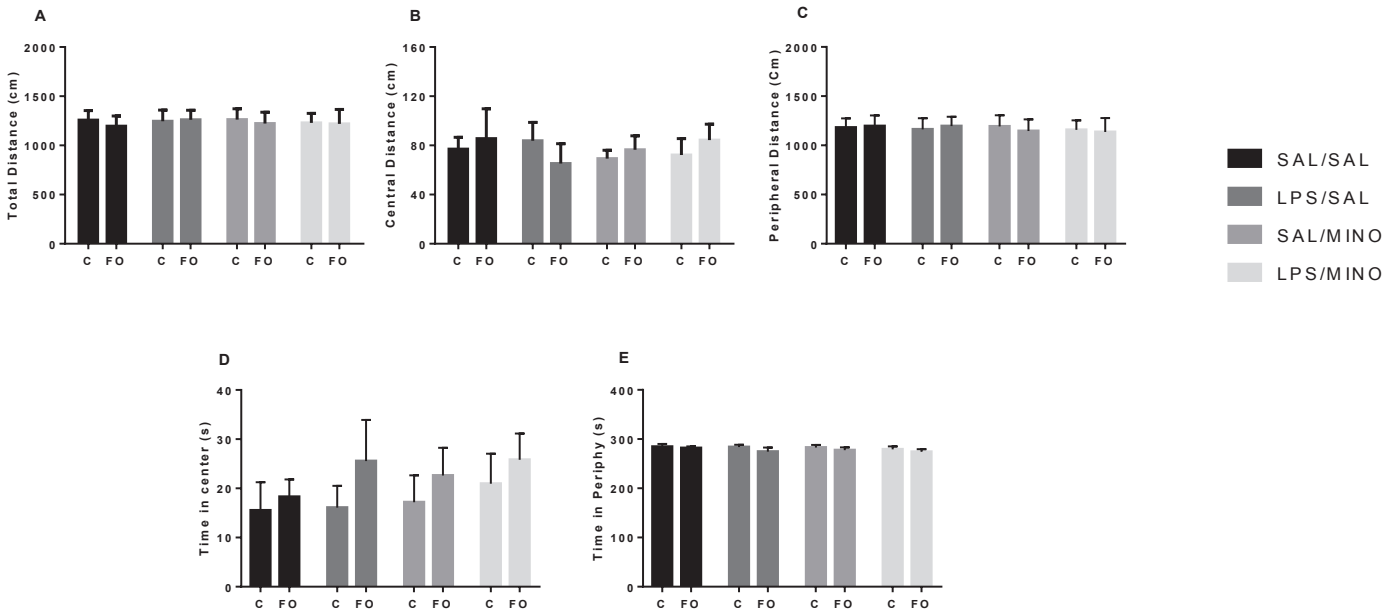


Fig 7. Open Field test. **A** Total Distance **B** Central distance **C** Peripheral distance **D** Time in center **E** Time in periphery. C: non-supplemented group (control); FO: Supplemented group. C/SAL/SAL (control group that received only saline injections, n=16); C/LPS/SAL (control group, with Lipopolysacharide and saline injections, n=16); C/SAL/MINO (control group, with injection of minocycline and saline, n=16); C/LPS/MINO (control group, with LPS and Minocycline injections, n=16). FO/SAL/SAL (supplemented, with saline injections, n=16); FO/LPS/SAL (supplemented, with Lipopolysacharide and saline injections, n=16); FO/SAL/MINO (supplemented, with saline and minocycline injections, n=15); FO/LPS/MINO (supplemented, with Lipopolysacharide and Minocycline injections, n=17). Three-way ANOVA. Values are expressed as mean± S.E.M.

*Modified Forced Swimming Test*

Regarding swimming (Fig. 8A), three-way ANOVA showed an effect of the fish oil supplementation [$F(1,120)= 71.93$; $p\leq0.00001$] and an interaction between LPS and Minocycline [$F(1,120)= 3.7$; $p\leq0.05$]. Tukey's *post hoc* test showed that all supplemented groups displayed higher frequency of this behavior compared to all non-supplemented groups ($p\leq0.04$). The C/LPS/SAL group presented lower swimming frequency compared to the other Control groups ($p\leq0.03$).

There was no effect of LPS [$F(1,120)=3.4$; n.s.], MINO [$F(1,120)= 2.1$; n.s.], and no interaction between: FO and LPS [$F(1,120)= 2.9$; n.s], FO and MINO [$F(1,120)= 2.16$; n.s] or between FO, LPS, and MINO [$F(1,120)= 2.7$; n.s].

For immobility (Fig. 8B), three-way ANOVA revealed an effect of supplementation [$F(1,120)=83.3$; $p\leq0.00001$], an effect of LPS [$F(1,120)=4.9$; $p\leq0,03$], an effect of MINO [$F(1,120)=23.62$; $p\leq0.00004$], and an interaction between FO and LPS [$F(1,120)=5.61$; $p\leq0.02$]. *Post hoc* test showed that LPS increased immobility -

C/LPS/SAL compared to all groups ($p \leq 0.008$). The two non-supplemented groups C/SAL/MINO and C/LPS/MINO exhibited higher immobility frequency compared to the supplemented groups FO/SAL/MINO and FO/LPS/MINO ($p \leq 0.05$) but not compared to FO/SAL/SAL and FO/LPS/SAL. All FO-groups presented less immobility frequency when compared to C/SAL/SAL and C/LPS/SAL ($p \leq 0.0001$).

There was no interaction between: FO and MINO [$F(1,120)=2.6$; n.s.], LPS and MINO [$F(1,120)=2.62$; n.s.] or FO, LPS and MINO [$F(1,120)=1.28$; n.s.]

Regarding climbing behavior (Fig. 8c) three-way ANOVA revealed an effect of MINO [$F(1,120)=17.52$; $p \leq 0.000005$] but *post hoc* test didn't show any differences among groups. There were no effects of supplementation [$F(1,120)=0.05$; n.s.] or LPS [$F(1,120)=0.099$; n.s.], and no interaction between: FO and LPS [$F(1,120)=0.63$; n.s.], FO and MINO [$F(1,120)=0.13$; n.s.], LPS and MINO [$F(1,120)=0.26$; n.s.] or FO, LPS and MINO [$F(1,120)=0.6$; n.s.]

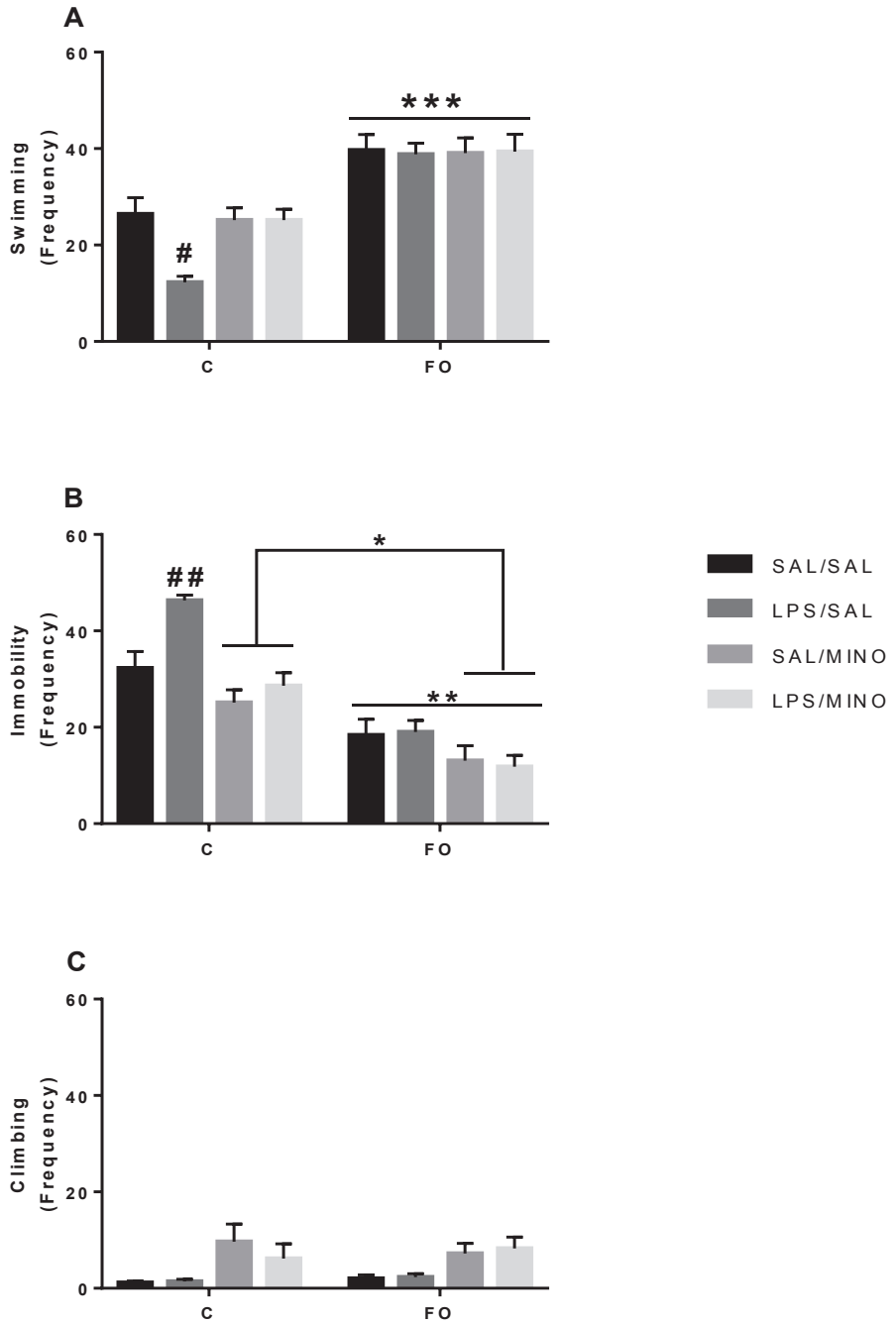


Fig. 8. Modified Forced Swim test. A Swimming; B Immobility; C Climbing. C: non-suplemented (control) groups FO: supplemented (fish oil) groups. Control/saline/saline (C/SAL/SAL, control group that received only saline injections, n=16); control/LPS/saline (C/LPS/SAL, control group, with Lipopolysacharide and saline injections, n=16); control/saline/minocycline (C/SAL/MINO, control group, with injection of minocycline and saline, n=16); control/LPS/Minocycicline (C/LPS/MINO, control group, with LPS and Minocycline injections, n=16). Fish Oil/saline/saline (FO/SAL/SAL, supplemented, with saline injections, n=16); Fish oil/LPS/saline (FO/LPS/SAL, supplemented, with Lipopolysacharide and saline injections, n=16); Fish oil/saline/minocycline (FO/SAL/MINO, supplemented, with saline and minocycline injections, n=15); fish oil/LPS/monocycline (FO/LPS/MINO, supplemented, with LPS and Minocycline injections). Three-way ANOVA followed by Tukey's post-hoc test. Values are expressed as mean± S.E.M. ***$p \leq 0.04$ compared to all non-supplemented groups; #$p \leq 0.03$ compared to all FO-groups and to C/SAL/SAL, C/SAL/MINO and C/LPS/MINO groups; **$p \leq 0.0001$ compared to C/SALSAL and C/LPS/SAL; ##$p \leq 0.008$ compared to the other C-groups and to all FO-groups. *$p \leq 0.05$.

*Neurochemical Data (HPLC)*

Figure 9 shows neurochemical quantification in the hippocampus of adult animals (5-HT is shown in Fig. 9A; 5-HIAA in Fig. 9B and the ratio 5-HIAA/5-HT, in Fig. 9C). Regarding 5-HT levels three-way ANOVA revealed an effect of fish oil supplementation [$F(1,38)=77,60$; $p\leq0.00001$], and an interaction between FO, LPS and MINO [$F(1,38)=3,73$; $p\leq0.05$]. *Post hoc* test showed that FO groups presented higher levels of 5-HT compared to all non-supplemented groups ($p\leq0.002$). The C/LPS/SAL group exhibited lower levels of serotonin compared to the other control-groups ($p\leq0.05$), including the C/LPS/MINO, showing that Minocycline, such as fish oil supplementation, is effective in preventing LPS effect.

There was no effect of LPS [$F(1,38)=2,42$; n.s.], MINO [$F(1,38)=3,2$; n.s.], or any interactions between FO and LPS [$F(1,38)=0,11$; n.s.], FO and MINO [$F(1,38)=0,06$; n.s.], or LPS and MINO [$F(1,38)=0,4$; n.s.].

Three-way ANOVA didn't show any differences in 5HIAA levels for FO [$F(1,38)=2,44$; n.s.], LPS [$F(1,38)=0,25$; n.s.]. MINO [$F(1,38)=0,04$; n.s.], or any interactions between FO and LPS [$F(1,38)=3,78$; n.s.], FO and MINO [$F(1,38)=0,96$; n.s.], LPS and MINO [$F(1,38)=0,26$; n.s.] or between FO, LPS and MINO [$F(1,38)=0,023$; n.s.].

Regarding the ratio 5-HIAA/5-HT three-way ANOVA showed an effect of fish oil [$F(1,38)=11,3$; $p\leq0.001$], and MINO [$F(1,38)=4,65$ $p\leq0.04$], and an interaction between FO and LPS [$F(1,38)=4,85$; $p\leq0.03$] and between FO, LPS and MINO [$F(1,38)=3,88$; $p\leq0.05$]. *Post hoc* test showed that the non-supplemented group that received only LPS (C/LPS/SAL) exhibited higher 5HIAA/5-HT ratio, compared to all other C- and FO-groups ($p\leq0,03$).

No effect was found for LPS [$F(1,38)=2,92$; n.s.], no interaction between FO and MINO [$F(1,38)=3,64$; n.s.] or LPS and MINO [$F(1,38)=3,0$; n.s.].

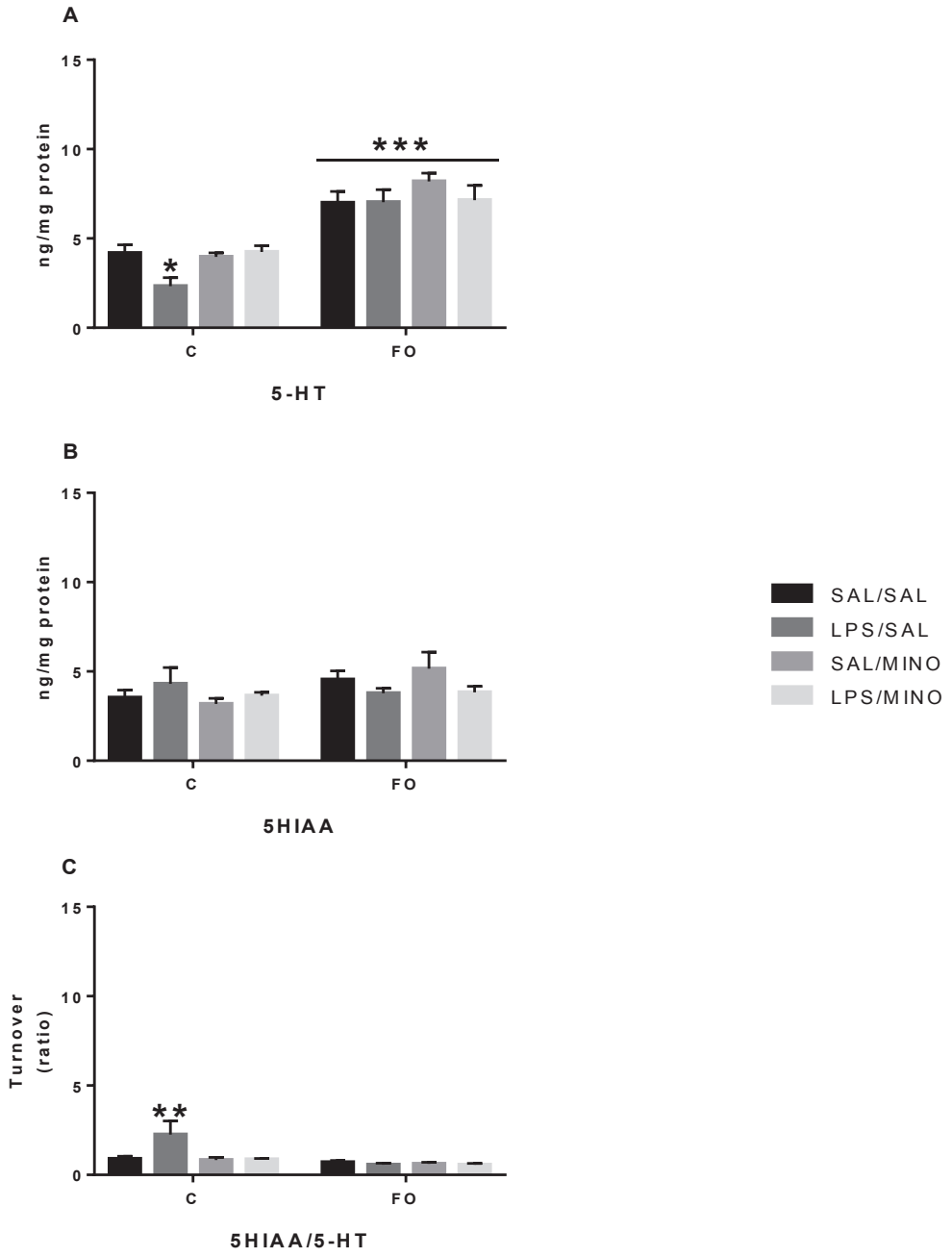


Fig 9. Hippocampal Neurochemical data. C: non-supplemented group (control); FO: Supplemented group. C/SAL/SAL(control group that received only saline injections, n=5); C/LPS/SAL (control group, with Lipopolysacharide and saline injections, n=5); C/SAL/MINO (control group, with injection of minocycline and saline, n=5); C/LPS/MINO (control group, with LPS and Minocycline injections, n=5); FO/SAL/SAL, (supplemented, with saline injections, n=4); FO/LPS/SAL (supplemented, with Lipopolysacharide and saline injections, n=5); FO/SAL/MINO (supplemented, with saline and minocycline injections, n=4); FO/LPS/MINO (supplemented, with LPS and Minocycline injections, n=5). Three-way ANOVA. Values are expressed as mean± S.E.M. *p ≤0.05 compared to the other control groups; ***p≤0.002 compared to all control groups; **p≤0.03 compared to all groups.

*Western Blot*

Fig 10 shows IDO expression in hippocampus. Three-way ANOVA revealed an effect of FO [F(1,30) = 3.4; p≤0.05], LPS [F(1,30) = 4.10; p≤0.05] and an effect of MINO [F(1,30) = 3.10; p≤0.05]. *Post hoc* test showed that the non-supplemented group who received LPS exhibited higher expression of IDO (C/LPS/SAL vs C/SAL/SAL, p≤0.05) and both supplementation and Minocycline prevented this effect (p≤0.05).

There were no interactions between: FO and LPS [F(1,30) = 1.54; n.s.], FO and MINO [F(1,30) = 2.3; n.s.], LPS and MINO [F(1,30) = 1.95; n.s.] or FO, LPS and MINO [F(1,30) = 2.2; n.s.].

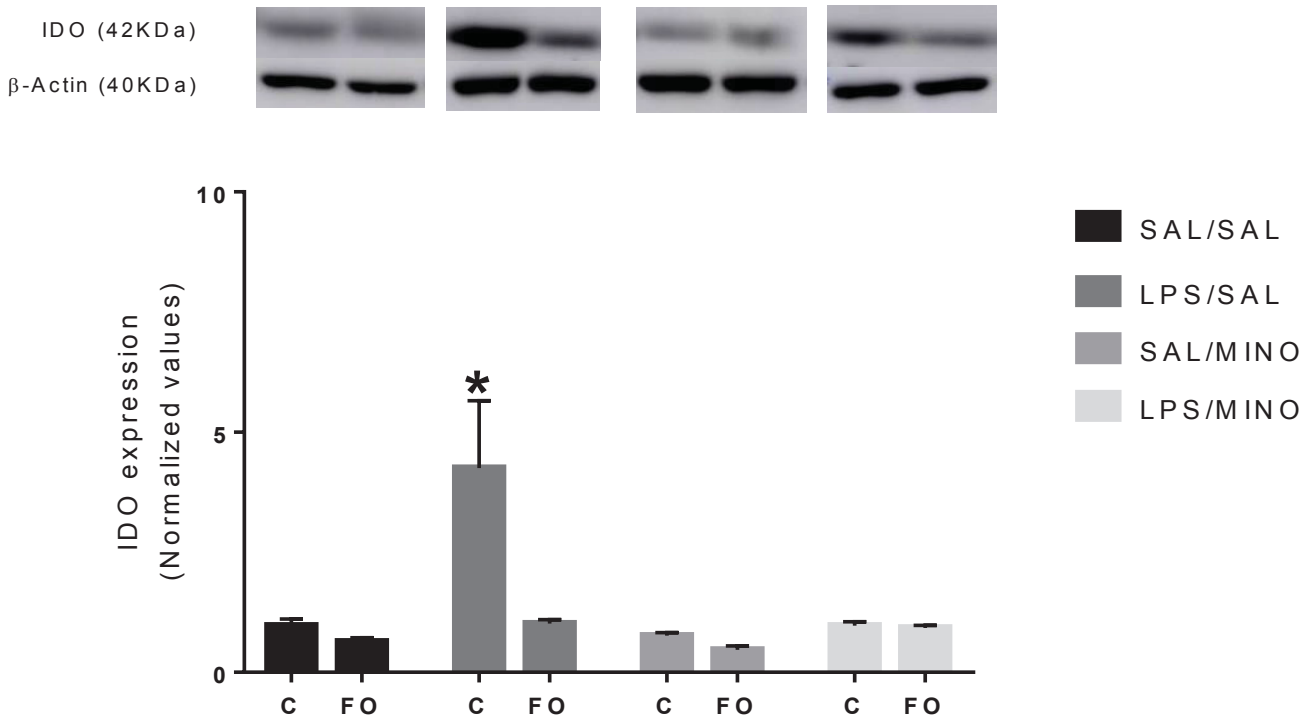


Fig 10 Western Blot analyses: IDO expression (normalized values). C: non-suplemented (control) groups FO: supplemented (fish oil) groups. C/SAL/SAL (control group that received only saline injections, n=5); C/LPS/SAL (control group, with Lipopolysacharide and saline injections, n=5); C/SAL/MINO (control group, with Minocycline and saline injections, n=5); C/LPS/MINO (control group, with LPS and MINO injections, n=5). FO/SAL/SAL (supplemented, with saline injections, n=4); FO/LPS/saline (supplemented, with Lipopolysacharide and saline injections, n=5); FO/SAL/MINO (supplemented, with saline and Minocycline injections, n=4); FO/LPS/MINO(supplemented, with LPS and Minocycline injections, n=5). Three-way ANOVA followed by Tukey's post-hoc test. Values are expressed as mean± S.E.M. *p≤0.05 compared to all groups.

## Discussion

The major findings of our study are that fish-oil supplementation promotes a strong antidepressant effect, related to increased levels of serotonin and decreased expression of IDO. These effects remained in LPS-injected animals, highlighting the importance of ômega-3 in prevent depressive-like states.

The LPS depression model, with a single systemic injection, is a widely employed tool to investigate depressive-like behavior in rodents, mimicking human depressive symptoms occurring in acute infectious diseases. LPS is the major component of the outer membrane of Gram-negative bacteria, and its injection provokes the physiological and behavioral changes called sickness behavior, which is mediated by pro-inflammatory cytokines. The sickness behavior is an adaptive response that enhances recovery by conserving energy to combat acute inflammation. There are considerable phenomenological similarities between sickness behavior and depression, for example, behavioral inhibition, anhedonia, fatigue, anorexia and weight loss (CUSTÓDIO et al., 2013; DANTZER et al., 2008; O'CONNOR et al., 2009a).

Previous studies found that the peak of sickness is about 6h after LPS injection, which is directly related to an increase in pro-inflammatory cytokines levels. 24 hours later these behavioral changes apparently disappears and depressive-like behavior is more prominent (FERREIRA MELLO et al., 2013; O'CONNOR et al., 2009a). In line with these findings, no difference was found in locomotor activity in open field test 24h after LPS injection in our experiments. The absence of locomotor changes indicates that the locomotor impairment from sickness behavior was not present.

On MFST, we observed a depressive-like behavior induced by LPS injection, seen by increased frequency of immobility. Immobility in this test is a typical behavior after immune challenge, demonstrated by a number of studies (DANG et al., 2017; DUNN, 2006; FERREIRA MELLO et al., 2013; O'CONNOR et al., 2009a).

One study showed that LPS model is more effective in inducing depressive-like behaviors when compared to Chronic Mild Stress, since mice that were injected with LPS exhibited greater immobility time on forced swim test and tail suspension test. These effects were related to higher levels of pro-inflammatory cytokines induced by

the immune challenge, and more prominent changes, such as lower levels of 5-HT and BDNF, were found in hippocampus (ZHAO et al., 2016).

There are evidence showing that the link between inflammation and depressive-like behavior is mediated by the enzyme indoleamine-2,3-dioxygenase. IDO is activated by pro-inflammatory cytokines, and it catabolizes tryptophan into kynurenines, leading to a reduction in serotonin levels (MAES et al., 2011b). It has been demonstrated that its inhibition with the competitive inhibitor 1-MT and with indirect inhibitor minocycline blocks the depressive-like behavior after LPS-induced acute inflammation (O'CONNOR et al., 2009a).

Minocycline is a semi-synthetic second generation tetracycline compound that effectively crosses the blood-brain barrier (YONG et al., 2004). It has been shown that minocycline have neuroprotective activity, which appears to be distinct from its antimicrobial activities (TIKKA et al., 2001). Also, it is a potent antioxidant and has a broad spectrum anti-inflammatory properties (KRAUS et al., 2005; MORIMOTO et al., 2005; SAPADIN; FLEISCHMAJER, 2006).

As expected, we found that pretreatment with Minocycline prevented LPS induced-depressive-like behavior, seen by normalization of immobility frequency in animals who received LPS and Minocycline. We found that this effect is related to IDO inhibition and consequently prevention of the drop in 5-HT levels (DA SILVA DIAS et al., 2016; O'CONNOR et al., 2009a). It has been suggested that this drug exerts its neuroprotective and psychotropic effects through indirect modulation of monoaminergic systems. One study found that minocycline was effective in attenuate the reductions in 5-HT and dopamine levels, such as density of both neurotransmitters transporters, after the administration of 3,4-Methylenodioxymethanphetamine (MDMA) (ZHANG et al., 2006). Also, the pretreatment with this tetracycline was effective in prevent the reduction in dopamine, serotonin and noradrenaline levels in rats treated with 3-nitropropionic acid (AHUJA; BISHNOI; CHOPRA, 2008).

In addition to IDO inhibition and consequently antidepressant effects, minocycline blocked the effects of sickness, seen by the prevention of weight-loss following LPS injection. However, this is not surprising, since minocycline acts very early blocking the events triggered by LPS, that starts with Toll-like receptor 4 activation. Its potent anti-inflammatory properties possibly antagonized the

inflammatory events after lipopolysaccharide injection (O'CONNOR et al., 2009b; SAPADIN; FLEISCHMAJER, 2006).

The tryptophan analog 1-methyltryptophan (1-MT) is a competitive inhibitor of IDO, and *in vitro* blocks human dendritic cell regulatory function and *in vivo* blocks IDO-mediated immune events in animal models of rheumatoid arthritis and multiple sclerosis, and has antidepressant effetcs on LPS depression model and in Type 1 diabetes model (DA SILVA DIAS et al., 2016; KWIDZINSKI et al., 2005; MUNN et al., 2002; O'CONNOR et al., 2009a; SEO et al., 2004). In line with these findings, we found that 1-MT blocked the depressive-like behavior after the imune challenge, which is related to the specific blockade of IDO activity (but not expression). However, 1-MT injection was not suficient to block the drop in 5-HT levels.

A possible explanation is that the tryptophan metabolites can play a role in depression, independently of serotonin. Since two of the compounds originated from Kynurenine pathway – 3-hydroxykynurenine and quinolinic acid – are neurotoxic and can directly induce depressive symptoms, 1-MT antidepressant activity could be related to inhibiton of these metabolites, through IDO inhibition (GUILLEMIN et al., 2007; SCHWARCZ, 2004).

There are evidence that conventional antidepressants, specially SSRIs, have anti-inflammatories properties, leading to a decrease in inflammatory markers and supressing IDO expression, in addition to inhibition of serotonin transporter (DA SILVA DIAS et al., 2016; GALECKI; MOSSAKOWSKA-WÓJCIK; TALAROWSKA, 2017; WIĘDŁOCHA et al., 2017) .

This fact highlight the idea that maybe these drugs acts through these two mechanisms, increasing serotonin levels in brain, pointing out to new estrategies with antidepressant and anti-inflammatory properties to prevent and treat depression, or even potentiate the conventional treatment. A promising way to achieve this goal is the suplementation with fish-oil, rich in omega-3.

The efficacy of fish oil supplementation in ameliorate depressive symptoms has been demonstrated by a number of clinical and pre-clinical studies (CARABELLI et al., 2014a; DA SILVA et al., 2008; MCNAMARA; STRAWN, 2013; PUDELL et al., 2013).

Previous works from our group found that fish-oil antidepressant-like effect was related to modulation of serotonin system, with $5HT_{1A}$ sensitization and increased

BDNF levels in hippocampus (CARABELLI et al., 2014b; VINES et al., 2012a). However, the question regarding these mechanisms remain unsolved. Here we hypothesized that omega-3 acts through inhibition of IDO, leading to a decrease in indoleamine 2,3-dioxygenase expression and consequently restoration of 5-HT levels.

As expected, we found an antidepressant effect of supplementation, seen by increased frequency of swimming and consequently lower immobility in modified forced swimming test. Also, fish-oil supplementation prevented LPS-induced depressive-like behavior, replicating previous findings (DANG et al., 2017; SHI et al., 2016).

Since IDO is over-expressed in LPS-injected animals, we investigated the expression of this enzyme in supplemented animals. We found that fish-oil decreased IDO expression, preventing its induction by LPS, confirming the link between antidepressant effect of this compound and decreased expression of IDO found in previous studies (DANG et al., 2017; SHI et al., 2016).

We tested the two IDO inhibitors previously mentioned – 1-MT and Minocycline – in supplemented animals injected with LPS, to confirm the hypothesis of an augmentation of antidepressant effect promoted by fish-oil supplementation. To our knowledge, this is the first study to investigate the effects of these drugs in fish-oil supplemented animals.

Surprisingly, we did not observe an aditional effect of 1-MT in supplemented animals. This could be explained by the fact that, possibly, omega-3 supressed IDO at a maximum level, and any additional treatment in attempt to specifically block IDO would be ineffective. The same could be happened with minocycline regarding swimming behavior. However, when we analyzed immobility frequency, we found an augmentation of fish oil effect promoted by the treatment with this antibiotic. This additional effect occurred through increasing climbing behavior - although not statistically significant - which could suggest an involvement of noradrenergic system in the mechanism of this drug. These data point out to another study, which found increased climbing behavior promoted by minocycline. Also, that study did not find a synergistic effect between fluoxetine and minocycline and we propose that the same ocurred with fish oil and minocycline, indicating that, differently of ômega-3, this tetracycline do not have direct effect on serotonergic system (MOLINA-HERNÁNDEZ et al., 2008b).

The neurochemical data showed that fish-oil supplementation blocked LPS-induced serotonin depletion and prevented the increased 5HIAA/5-HT ratio in LPS-animals. However, all FO groups presented higher levels of serotonin, even the group who received only saline. This data indicates that omega-3 increases serotonin in different ways.

Patrick and Ames (2015) proposed that the W-3 PUFAS Eicosapentaenoic Acid (EPA) and Docosahexaenoic acid (DHA) modulate serotonin function by different mechanisms. The $E_2$ series prostaglandins, generated from arachidonic acid - an omega-6 fatty acid produced from linoleic acid – inhibits serotonin release. EPA inhibits the formation of arachidonic acid and, consequently, inhibits the formation of $E_2$ series prostaglandins in young and adult individuals. Given the fact that EPA inhibits $E_2$ series prostaglandins, it seems plausible that EPA in the brain would be important for normal serotonin release (GÜNTHER et al., 2010; PATRICK; AMES, 2015; REES et al., 2006; SCHLICKER; FINK; GÖTHERT, 1987; VEDIN et al., 2010).

In this sense, the anti-inflammatory properties of omega-3 are still linked to its antidepressant effect. In fact, we found that long-term supplementation attenuated 24h-weight loss followed by LPS injection, pointing to the anti-inflammatory effects of this compound, with decrease in pro-inflammatory cytokines levels during acute phase response, antagonizing LPS effect in a similar way to minocycline, although less effective (SHI et al., 2016) .

Another mechanism proposed is the DHA-mediated regulation of serotonin receptor function, which depends on cell membrane fluidity. As the membrane becomes less fluid, the binding of serotonin to its receptor decreases significantly. DHA composition in the lipid membrane is necessary for adequate membrane fluidity, and it increases the sensitivity of serotonin receptors (BRADBURY, 2011; CALDER, 2012; HERON et al., 1980; PAILA; GANGULY; CHATTOPADHYAY, 2010; WASSALL; STILLWELL, 2009).

These mechanisms, in addition to IDO inhibition, could explain the effects of fish oil supplementation in prevent and alleviate depressive symptoms, through increase of 5-HT levels and action.

In conclusion, we showed that supplementation with fish oil suppressed LPS-induced IDO expression, like minocycline. Our neurotransmitter data suggest that another mechanism beyond IDO is involved. However, further studies are necessary

to elucidate the exact mechanisms by which omega-3 increases serotonin levels and exerts its antidepressant effect.

## 6 DISCUSSÃO

Entre os principais achados desse trabalho destacamos que a suplementação com óleo de peixe promoveu um forte efeito antidepressivo, que está relacionado com uma diminuição na expressão da enzima IDO e aumento nos níveis de serotonina. Esse efeito permaneceu nos animais que receberam LPS, mostrando a importância da suplementação com ômega-3 em prevenir a ocorrência de episódios depressivos.

O modelo de depressão do LPS, com uma única injeção sistêmica, é uma ferramenta amplamente utilizada para investigação do comportamento tipo-depressivo em roedores, mimetizando os sintomas depressivos ocorridos em pessoas portadoras de doenças com características inflamatórias. O LPS é o principal componente da membrana de bactérias gram-negativas, e a injeção deste é capaz de provocar alterações fisiológicas e comportamentais coletivamente chamadas de comportamento de doença, mediado por citocinas pró-inflamatórias. Esse comportamento é uma resposta adaptativa com o objetivo de melhorar a recuperação e conservar energia para combater o agente infeccioso e diminuir a inflamação. Existem similaridades fenomenológicas consideráveis entre o comportamento de doença e a depressão, como por exemplo, a inibição comportamental, anedonia, fadiga, anorexia e perda de peso (CUSTÓDIO et al., 2013; DANTZER et al., 2008; O'CONNOR et al., 2009a).

Estudos apontam que o pico dessas mudanças comportamentais acontece cerca de 6 horas após a injeção do LPS, o que está relacionado com o pico de secreção de citocinas pró-inflamatórias. Vinte e quatro horas após, essas mudanças desaparecem e o comportamento tipo-depressivo fica mais evidente (FERREIRA MELLO et al., 2013; O'CONNOR et al., 2009a).

Nesse trabalho, não observamos diferenças na atividade locomotora no teste do campo aberto 24h após a injeção do LPS, o que indica que o déficit locomotor, característico do comportamento de doença, não estava mais presente.

Corroborando estudos prévios, observamos um comportamento tipo-depressivo induzido pelo LPS, visto pela maior frequência de imobilidade no teste da natação forçada modificado. Este é um comportamento típico após o desafio imunológico, demonstrado por vários estudos (DANG et al., 2017; DUNN, 2006; FERREIRA MELLO et al., 2013; O'CONNOR et al., 2009a).

Um estudo demonstrou que o modelo do LPS é mais eficiente em induzir um comportamento tipo-depressivo, quando comparado com o estresse crônico moderado, uma vez que camundongos injetados com o lipopolissacarídeo apresentaram maior tempo de imobilidade nos testes de suspensão pela cauda e da natação forçada. Estes efeitos estão relacionados com a habilidade do LPS em induzir uma inflamação de forma mais agressiva, com maiores níveis de citocinas pró-inflamatórias. Entre as estruturas mais afetadas pela injeção do lipopolissacarídeo, destaca-se o hipocampo, que é extremamente sensível à mudanças provocadas pelo desafio imunológico. Foi demonstrado que uma única injeção de LPS em camundongos provoca mudanças nos níveis de serotonina e BDNF apenas nesta estrutura, sem mudanças no cortex pré-frontal e estriado (ZHAO et al., 2016).

Muitas evidências apontam que a relação entre a inflamação e o comportamento tipo-depressivo é mediada pela enzima indoleamina-2,3-dioxigenase, que é ativada por citocinas pró-inflamatórias. Esta enzima cataboliza o triptofano na via das quinureninas e leva a uma redução nos níveis de serotonina, provocando os sintomas depressivos (MAES et al., 2011c).

Tem sido demonstrado que a inibição da IDO, seja pelo inibidor indireto minociclina ou pelo inibidor competitivo 1-MT, bloqueia o comportamento-tipo depressivo induzido pelo LPS (O'CONNOR et al., 2009a).

A minociclina é um antibiótico semi-sintético de segunda geração pertencente à família das tetraciclinas, capaz de atravessar a barreira hemato-encefálica (YONG et al., 2004). Esta droga apresenta atividade neuroprotetora, anti-inflamatória e antioxidante, e estas características são independentes da atividade antimicrobiana (KRAUS et al., 2005; MORIMOTO et al., 2005; SAPADIN; FLEISCHMAJER, 2006; TIKKA et al., 2001).

Como esperado, o pré-tratamento com minociclina preveniu o comportamento tipo-depressivo induzido pelo LPS, visto pela normalização da frequência de imobilidade dos animais que receberam os dois tratamentos. Este efeito está relacionado com a supressão da enzima IDO e prevenção da queda de serotonina encontrados nesse trabalho, assim como encontrado em estudos anteriores (DA SILVA DIAS et al., 2016; O'CONNOR et al., 2009a). Foi sugerido que essa droga exerce seus efeitos neuroprotetores e psicotrópicos através da modulação indireta do sistema monoaminérgico. Um estudo demonstrou que a minociclina foi eficaz em

atenuar a redução de serotonina e de dopamina, assim como a densidade dos transportadores de ambos os neurotransmissores após administração de 3,4-metilenodioximetanfetamina (MDMA) (ZHANG et al., 2006). Ainda, o pré-tratamento com essa tetraciclina também se mostrou eficaz em prevenir a diminuição de dopamina, serotonina e noradrenalina em ratos tratados com ácido 3-nitropropiônico (AHUJA; BISHNOI; CHOPRA, 2008).

Em nosso trabalho, a minociclina também foi capaz de bloquear uma característica típica do comportamento de doença, que é a perda de peso. Isso não é surpreendente, uma vez que essa droga age no início da cadeia de eventos provocados pelos LPS, começando com a ativação do receptor Toll-like 4. Possivelmente, esses efeitos anti-inflamatórios antagonizaram os efeitos do LPS, sendo responsáveis por inibir o comportamento tipo-depressivo (O'CONNOR et al., 2009b; SAPADIN; FLEISCHMAJER, 2006).

No presente estudo, encontramos que o 1-MT, um inibidor competitivo da IDO, foi capaz de prevenir o efeito depressivo provocado pelo LPS, replicando os achados de outros estudos (DA SILVA DIAS et al., 2015; O'CONNOR et al., 2009b).

Esse efeito está relacionado com o bloqueio específico da IDO, impedindo sua atividade, porém sem alterações na expressão dessa enzima. No entanto, o 1-MT não foi capaz de reestabelecer os níveis de serotonina no hipocampo dos animais. Uma possível explicação reside no papel dos metabólitos do triptofano na patogênese da depressão. O metabolismo do triptofano pela IDO dá origem à Kinurenina que, por sua vez, origina compostos formados em etapas subsequentes por diferentes enzimas. Entre esses compostos destacam-se a 3-hidroxikinurenina e o ácido quinolínico. Esses dois compostos são capazes de gerar radicais livres, e têm atividade neurotóxica, sendo capazes de, diretamente, induzir sintomas depressivos, independentemente da redução dos níveis de serotonina (DANTZER et al., 2011b). Em um estudo foi demonstrado que a administração de L-kinurenina induziu um comportamento tipo-depressivo dose-dependente em camundongos (O'CONNOR et al., 2009a). O ácido quinolínico é um agonista do receptor glutamatérgico NMDA, e foi sugerido que a disfunção glutamatérgica em alguns indíviduos com transtornos de humor possa ser consequência de níveis anormais desse composto, contribuindo para a excitotoxicidade. Desta forma, observa-se a importância da neurotransmissão glutamatérgica na etiologia da depressão (GUILLEMIN et al., 2007; MYINT, 2012;

SCHWARCZ, 2004; STEINER et al., 2012). Ainda que insuficiente para prevenir a queda nos níveis de serotonina, o bloqueio da IDO pelo 1-MT pode ter sido suficiente para diminuir os níveis desses metabólitos, de forma a promover o efeito antidepressivo visto no teste da natação forçada.

Destacando a importância da inflamação na fisiopatologia da depressão, existem evidências de que os antidepressivos, em especial os inibidores seletivos da recaptação da serotonina, possuem atividade anti-inflamatória, levando à diminuição da expressão da IDO. Isto poderia explicar, em parte, seu efeito antidepressivo, em adição à inibição do transportador da serotonina (DA SILVA DIAS et al., 2016; GALECKI; MOSSAKOWSKA-WÓJCIK; TALAROWSKA, 2017; WIĘDŁOCHA et al., 2017) .

No entanto, esse tratamento não é totalmente eficaz e cerca de 50% dos pacientes não respondem de forma adequada e os efeitos colaterais provocados em parcela significativa dos pacientes faz com que a adesão ao tratamento seja ainda menor (FAVA et al., 2008; TRIVEDI et al., 2006b). Nesse sentido, muitos estudos clínicos e pré-clínicos apontam a eficácia da suplementação com óleo de peixe em melhorar os sintomas depressivos, o que faz desse composto uma importante alternativa para a prevenção e tratamento da depressão (CARABELLI et al., 2014a; DA SILVA et al., 2008; MCNAMARA; STRAWN, 2013; PUDELL et al., 2013).

Estudos prévios de nosso grupo mostraram que o efeito antidepressivo da suplementação com óleo de peixe está relacionado com um aumento nos níveis de serotonina, sensibilização do receptor 5-$HT_{1A}$ e aumento de BDNF no hipocampo de ratos Wistar (CARABELLI et al., 2014a; VINES et al., 2012a). No entanto, os mecanismos pelos quais o ômega-3 aumenta a serotonina permanecem sem resposta. Nesse trabalho, nós hipotetizamos que ômega-3 exerce seu efeito antidepressivo através da inibição da IDO, diminuindo sua expressão e restaurando os níveis de serotonina.

Como esperado, observamos um efeito antidepressivo promovido pela suplementação com óleo de peixe, visto pelo aumento na frequência de natação e menor frequência de imobilidade no teste da natação forçada modificado. Ainda, esse efeito permaneceu em animais que receberam injeção de LPS, confirmando a eficácia da suplementação em prevenir o efeito tipo-depressivo após o desafio imunológico, como demonstrado por trabalhos anteriores (DANG et al., 2017; SHI et al., 2016).

Esse efeito está relacionado com a menor expressão da IDO encontrada nos animas suplementados que receberam LPS, permanecendo em níveis semelhantes ao grupo controle que recebeu salina. Uma vez confirmada a eficácia do ômega-3 em inibir a IDO, investigamos os efeitos das drogas previamente citadas – 1-MT e Minociclina – juntamente com o óleo de peixe.

Ao nosso conhecimento, esse é o primeiro estudo que investigou a eficácia dessas drogas em animais suplementados com ômega-3, com objetivo de verificar aumento no efeito antidepressivo.

Devido à inibição da IDO pelo 1-MT e minociclina, além dos efeitos protetores desta última sob as vias serotoninérgicas, esperávamos um aumento no comportamento de natação promovido pelo óleo de peixe, uma vez que este comportamento está relacionado com um aumento de 5-HT. Surpreendentemente, não observamos nenhum efeito adicional do tratamento com os inibidores da IDO em animais suplementados com óleo de peixe nesse parâmetro. Uma hipótese e possível explicação para a ausência de efeito sinérgico entre o 1-MT e óleo de peixe seria o fato de que a suplementação, através de seu efeito anti-inflamatório, tenha causado máxima supressão da IDO e qualquer tratamento adicional com esse mesmo mecanismo seria inefetivo. O mesmo pode ter acontecido com a minociclina em relação à natação. Em relação à imobilidade, os animais suplementados que receberam tratamento com minociclina apresentaram frequência ainda menor desse comportamento. Esse efeito aditivo proporcionado pela minociclina se deu através do aumento do comportamento de escalada, e, embora não estatisticamente significativo, sugere um envolvimento no sistema noradrenérgico promovido por esta droga. Nossos dados apontam para outro estudo, o qual observou efeito da minociclina sob o comportamento de escalada e não encontrou efeito sinérgico do tratamento com fluoxetina e minociclina (MOLINA-HERNÁNDEZ et al., 2008b). Nós sugerimos que o mesmo tenha acontecido com o óleo de peixe e minociclina, indicando que esse antibiótico não tem ação direta sob o sistema serotoninérgico, diferentemente do óleo de peixe.

Em relação aos efeitos da suplementação isoladamente, os dados neuroquímicos apontam que o óleo de peixe aumentou os níveis de serotonina no hipocampo, replicando outros estudos de nosso grupo (CARABELLI et al., 2014b; VINES et al., 2012b). Além disso, a suplementação preveniu o aumento da razão

5HIAA/5-HT induzida pelo LPS. No entanto, o grupo suplementado que recebeu apenas injeções de salina também apresentou níveis de serotonina elevados. Uma vez que a expressão da IDO em animais não-estressados e sem nenhum outro tratamento é baixa, sem atividade significativa, esse dado indica que outro mecanismo, além da inibição da IDO, está envolvido no efeito antidepressivo do ômega-3 (DANTZER et al., 2011b; O'CONNOR et al., 2009b).

Patrick e Aimes (2015) propuseram que o ácido eicosapentaenoico (EPA) e o ácido docosahexaenóico (DHA) modulam os níveis de serotonina através de diferentes mecanismos. As prostaglandinas da série E2, geradas a partir do ácido araquidônico (ácido graxo poli-instaurado da família ômega-6, originado a partir do ácido linoleico), inibem a liberação de serotonina. Uma vez que o EPA inibe o metabolismo do ácido araquidônico e, consequentemente, a formação de prostaglandinas E2, pode-se concluir que o EPA é importante para a liberação normal de serotonina no encéfalo (PATRICK; AMES, 2015). Nesse sentido, frente a um desafio imunológico ou estresse comportamental, o EPA teria papel importante, causando um aumento da liberação de serotonina.

Outro mecanismo proposto é a regulação da função dos receptores serotoninérgicos induzida pelo DHA, que é dependente da fluidez da membrana. Se a membrana se torna menos fluida, a ligação da serotonina a seu receptor diminui significativamente. Logo, a presença do DHA na composição na membrana lipídica é necessária para maior fluidez, aumentando a sensibilidade dos receptores serotoninérgicos (BRADBURY, 2011; CALDER, 2012; HERON et al., 1980; PAILA; GANGULY; CHATTOPADHYAY, 2010; WASSALL; STILLWELL, 2009).

Esses mecanismos, em adição à inibição da IDO, explicariam o aumento de serotonina e efeito antidepressivo induzido pela suplementação com óleo de peixe.

Em resumo, mostramos que a suplementação com óleo de peixe levou à supressão da enzima IDO em animais que receberam LPS, e esse efeito se deu de forma semelhante à minociclina. Os dados neuroquímicos sugerem que outros mecanismos além da inibição da IDO estão envolvidos. Entretanto, mais estudos são necessários para elucidar os mecanismos exatos pelos quais o ômega-3 aumenta os níveis de serotonina e exerce seu efeito antidepressivo.

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# DOUTORADO SANDUÍCHE

**Os resultados nessa seção foram obtidos durante o período de intercâmbio, sob a orientação do Dr. Irwin Lucki, na Uniformed Services University of the Health Sciences (USUHS) – Bethesda, Maryland, EUA.**

**Curitiba, dezembro de 2017**

**UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES**
DEPARTMENT OF PHARMACOLOGY & MOLECULAR THERAPEUTICS
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September 18, 2017

RE: Bruno Carabelli

To whom it may concern:

Bruno Carabelli was funded by the CAPES Foundation to conduct part of his doctoral research from April 14 - July 31, 2017 in my laboratory at the Uniformed Services University in Bethesda MD USA. This was a highly successful internship. Bruno generated some very interesting pharmacological findings using a new stress model in mice. This work will be presented at the Society for Neuroscience meeting and contribute to a future publication.

Specifically, Bruno proposed to do a series of pharmacological experiments to determine whether novel fast-acting antidepressants reversed behavioral deficits relevant to depression following immune challenge with an endotoxin, lipopolysaccharide (LPS). These studies complemented both Bruno's doctoral work on the role of immunity in affective behaviors and the research interests of the laboratory, which focus on defining the biological mechanisms underlying the behavioral effects of drugs used in the treatment of psychiatric disorders.

First, Bruno characterized the physiological and behavioral changes promoted by immune challenge with LPS in mice. He confirmed that systemic administration of LPS resulted in pronounced sickness behavior as reflected by: hypothermia, decreased nesting behavior and increased kaolin intake (pica behavior) during the first 8 hours following immune challenge. When studied at 24 h post LPS, Bruno discovered that mice treated with LPS exhibited anhedonia, as measured by reduced sucrose preference and increased passive immobility in the forced swim test. Bruno then determined that prophylactic administration of low dose ketamine, a NMDA receptor antagonist, and low dose buprenorphine, a mixed opioid analgesic, reversed these behavioral deficits. Ketamine and buprenorphine rapidly alleviate symptoms in severely depressed patients and those resistant to treatment with conventional antidepressant therapy. The precise neurobiological correlates underlying the rapid action of these pharmacological different compounds is unknown. Therefore, Bruno investigated potential a convergent signaling pathway that could mediate the beneficial effects of these two compounds. Using western blot analysis, Bruno found elevated levels of ERK in the hippocampus of LPS treated mice that were attenuated by drug treatment. Based on Bruno's findings, work is ongoing in the lab to evaluate the role of the ERK signaling pathway in the anti-stress effects of novel antidepressant drugs.

On a personal level, Bruno was a delightful addition to the lab. He is a bright, energetic and highly skilled young scientist. He was dependable, creative and had an admirable work ethic. During the summer, Bruno's also showed exceptional ability as a mentor and teacher, as he carefully explained difficult concepts and demonstrated molecular and behavioral techniques to undergraduate and high school interns. At our lab meetings Bruno gave excellent presentations and provided helpful feedback and constructive criticism to other researchers. Ultimately Bruno wants to become an independent investigator. I am convinced that he has the academic and personal skills to be successful and to achieve his goal to be an independent scientist.

Sincerely,

Irwin Lucki

Irwin Lucki, Ph.D.
Professor and Chair

UNIVERSIDADE FEDERAL DO PARANÁ
SETOR DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE FISIOLOGIA
Laboratório de Neurofisiologia

Curitiba, 13 de outubro de 2017.

**À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes)**

Bruno Carabelli realizou parte dos seus experimentos de doutorado no laboratório do Dr. Irwin Lucki - Uniformed Services University in Bethesda MD USA – no período de 14 de abril a 31 de julho do corrente ano através do Edital de Seleção para bolsas PDSE/CAPES.

Os experimentos realizados por Bruno no Laboratório de Neurofisiologia no Brasil envolveram o estudo da neurobiologia da depressão utilizando o modelo da neurotoxina LPS(Lipopolissacarídeo bacteriano). Dr. Lucki, renomado cientista, investiga o efeito de drogas usadas no tratamento de distúrbios psiquiátricos sobre o comportamento depressivo. Assim, baseado nos interesses científicos em comum, o estabelecimento de colaboração entre o Laboratório de Neurofisiologia e um Laboratório consolidado pelo reconhecimento científico possibilitou o desenvolvimento de um intercambio que permeou a ampliação de publicações conjuntas e troca de experiências científicas.

Durante o período transcorrido nos EUA, acompanhei o trabalho de Bruno e seu empenho na realização de suas metas num período tão exíguo. Bruno mostrou-se competente, responsável e interessado em sua pesquisa, além disso a avaliação do professor responsável foi altamente positiva.

Diante destes fatos enfatizo que esta oportunidade de colaboração, propiciou acesso a um centro de excelência cuja realidade tecnológica e financeira encontra-se muito distante daquela vivenciada em nosso país e ampliou a análise crítica e maturidade científica do aluno.

Avalio como extremamente positiva a participação de Bruno Carabelli.

Atenciosamente,

Profa. Dra. Anete Curte Ferraz

# INVESTIGATION OF TWO FAST-ACTING ANTIDEPRESSANTS IN A MOUSE MODEL OF DEPRESSION WITH IMMUNE CHALLENGE (LPS).

# 1 Introduction

Converging lines of evidences suggest that depression is accompanied by activation of immuno-inflammatory pathways (MAES, 1993, 1995; SEIDEL et al., 1995; SLUZEWSKA et al., 1996). Accordingly, serum levels of inflammatory cytokines, for example, tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and IL-1 beta (IL-1b), are elevated in subjects with Major Depressive Disorder (DOWLATI et al., 2010; SEIDEL et al., 1995; SLUZEWSKA et al., 1996). The reason why this occurs is unclear, but evidence suggests that pathways between negative moods and inflammation are bi-directional because negative moods activate peripheral physiologic mechanisms that result in an up regulation of systemic levels of inflammation and conversely, peripheral inflammatory mediators signal the brain to affect behavioral, affective and cognitive changes that are consistent with symptoms of major depressive disorder (MESSAY; LIM; MARSLAND, 2012). Indeed, recent evidence point towards increased IgM and IgA responses directed against lipopolysaccharide (LPS) from gram negative gut commensals in patients with chronic depression (MAES et al., 2012).

These experimental observations in humans parallels preclinical developments in this field (DUNN; SWIERGIEL; DE BEAUREPAIRE, 2005). Accordingly, the systemic administration of LPS causes time-dependent behavioral alterations: sickness behavior is more evident approximately 6 h following LPS administration (i.e., along with the peak release of cytokines) (HUANG et al., 2008) while depressive-like behavior is observed 24 h after LPS challenge (PAINSIPP et al., 2011; SABINO et al., 2013).

The purpose of this study was to determine whether novel fast-acting antidepressants can reverse the depressive behavior produced by LPS challenge. To assess the depressive-like behavior promoted by immune challenge, male C57BL/6J mice received LPS intraperitoneally and 24h were submitted to the open field test and forced swim test. Two different novel fast-acting antidepressant drugs were tested: ketamine- a NMDA receptor antagonist – and buprenorphine – a kappa opioid receptor antagonist. These drugs were administered 1 h prior to LPS injection (25h before the behavioral tests).

The importance of conducting this study is due to the urgent need to understand the mechanism of action of novel drugs, like ketamine and buprenorphine, that have a fast onset of action. These drugs are capable of improving patients with

severe depression and also those patients refractory to the treatment with current first-line drugs. The current study will evaluate whether these drugs are able to produce antidepressant-like effects when depressive behavior has been caused by LPS, an inflammatory challenge.

## 2 Material and Methods

### 2.1 Animals

Male C57BL/6J mice (7 weeks old) were housed 5 per cage and maintained under a 12-h light cycle (lights on at 06:00 h) with room temperature of 22 ± 1 ◦C. Food and water were provided ad libitum before the tests. Food and water intake were measured during 8h following LPS injection and overnight. All procedures were carried out in accordance the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 2.2 LPS

LPS (Escherichia coli, O111:B4, Sigma Aldrich, Catalogue # L2630) were dissolved in saline and administered intraperitoneally in a dose of 0.83mg/Kg 24h before the behavioral tests.

### 2.3 Behavioral Scores

Mice were observed each hour, during 8 hours after LPS injection. Scores were assigned according to table 1. The rectal temperature was measured at the same time-points. Here we show only the data from the time point 8h.

| **SCORE SHEET** | |
|---|---|
| *Lack of activity (0-4)* | |
| 0 | Normal locomotor activity including walking/running and climbing behaviours |
| 1 | Slightly less than normal activity, including interrupted locomotion, traversing smaller distances, or, slower gait |
| 2 | Reduced activity, little locomotion |
| 3 | Reduced activity, no locomotion, head movements only, including sniffing and nosing behaviour |
| 4 | Substantially reduced activity, no body or head movements |
| *Provoked Activity (0-4)* | |
| 0 | Locomotor activity (avoidance walk/run) when nudged |
| 1 | Locomotor activity over short distance when nudged (less than ½ cage length) |
| 2 | No locomotion but startle response |
| 3 | No locomotion, no startle response, but locomotion when placed in the centre of cage (within 30 seconds of displacement) |
| 4 | No locomotion when gently placed in the centre of the cage (within 30 seconds of displacement) |
| *Hunching/position (0-5)* | |
| 0 | Normal position, no hunching |
| 1 | Slight hunch or uncomfortable position |
| 2 | Pronounced hunch/arching of back or slight hunch in a splayed position |
| 3 | More pronounced hunching –back legs tucked in under body or splayed |
| 4 | Substantial hunching – back legs under body, head tucked closely in to body |
| *Pilorection (0-2)* | |
| 0 | Normal |
| 1 | Distinct pilorection, e.g. half of the coat |
| 2 | Complete pilorection, coat standing on end, fluffed up |
| *Eye position (0-2)* | |
| 0 | Open and observing |
| 1 | Half closed |
| 2 | Closed |
| *Eye discharge (0-2)* | |
| 0 | None |
| 1 | Discharge from one eye (white/pink mucous over eye) |
| 2 | Discharge from both eyes |
| *Abnormal respiration (0-4)* | |
| 0 | Normal |
| 1 | Any slight deviation in respiration rate, quicker or slower |
| 3 | More pronounced respiration rate alterations from normal, speed and depth of breathing |
| 4 | Respiration difficulties, irregular respiratory rate or gasping behaviour |

## 2.4 Nesting Behavior

Nesting behavior was observed as a measure of well-being. The protocol is described below:

**Nesting behavior protocol**

1. Arrange 1 cage with standard bedding per test animal on wire racks in room G-114, equip each cage with a fresh nest pad and label cages 1 through *n*. Each cage should have water and a small amount of food.
2. Prepare drugs and vehicle.
3. Transport animals to room G-114 and weigh animals and re-label tails if needed.
4. Prepare injections at 10 mL/kg for each particular animal.
5. For each animal, inject with proper drug/vehicle, dosage, and volume and put directly into prepared cage corresponding to subject number. Record time of each injection. Each mouse will be housed singly for the duration of the experiment.
6. Find median time of injections to call “start time” and return each hour to score nesting behavior (see table). Halfway scores (e.g. 3.5) should be used as necessary. Lighting should correspond to the standard colony room light cycle.
7. After 8 scores, return mice to home cage and to colony room.

From *Deacon (2012) Assessing Burrowing, Nest Construction, and Hoarding in Mice. Journal of Visualized Experiments, 59:4-8.*

(DEACON, 2012)

| Score | Criteria | Example |
|---|---|---|
| **1** | The Nestlet is largely untouched (>90% intact) |  |

| | | |
|---|---|---|
| **2** | The Nestlet is partially torn up (50-90% remaining intact) |  |
| **3** | The Nestlet is mostly shredded but often there is no identifiable nest site: < 50% of the Nestlet remains intact but < 90% is within a quarter of the cage floor area, i.e. the cotton is not gathered into a nest but spread around the cage. Note: the material may sometimes be in a broadly defined nest area but the critical definition is that 50-90% has been shredded. |  |
| **4** | An identifiable, but flat nest: > 90% of the Nestlet is torn up, the material is gathered into a nest within a quarter of the cage floor area, but the nest is flat, with walls higher than mouse body height (curled up on its side) on less than 50% of its circumference. |  |

| | | |
|---|---|---|
| **5** | A (near) perfect nest: > 90% of the Nestlet is torn up, the nest is a crater, with walls higher than mouse body height on more than 50% of its circumference. |  |

## 2.5 Pica Behavior

The Pica behavior, which consist in the consumption of a substance without a nutritional value, was measure through kaolin intake. For habituation, kaolin pellets were provided *ad libitum* for one week prior the experiments.

Here we analyzed kaolin intake as part of sickness (malaise and nausea) induced by LPS (Figure 1).

Chow was provided *ad libitum* and its consumption was measured 8h and 24h after LPS injection, such as kaolin. For experiment 2, we show the consumption data for kaolin and chow 24h after LPS. Data is presented as percentage of total intake.

Figure 1. Kaolin pellets

## 2.6 Open Field Test

The open field apparatus (Stoelting) was 40 cm × 40 cm with opaque walls, illumination at ~700 lux. Each open field had an overhead camera connected to a computer with Any-Maze tracking software (Stoelting). Mice were individually placed into the center of the apparatus, and the software recorded movements of the animals for a 10 min session.

## 2.7 Sucrose Preference Test

Mice were individually housed. 24h before LPS injection, they were exposed to two 50mL bottles: one filled with water and the other with 2% sucrose. One day later, the amount consumed was measured as baseline. After LPS injection, bottles were filled up again and placed on cages in a switched position to avoid bias. After 24h the consumption was measured. Data were normalized to baseline and presented as % of total intake.

## 2.8 Forced Swimming Test

Mice were placed individually into glass cylinders (height 30 cm, diameter 21 cm) containing 15 cm water maintained at 25 °C ± 1°C. Water was renewed before test. Forced swimming test (FST) lasted for 6 min, and mice were immediately returned to their home cage. The total time spent immobile was scored.

A mouse was judged to be immobile when it floated in an upright position and made only small movements to keep its head above water.

## 2.9 Experimental Design

### 2.9.1 Experiment 1

In order to assess the antidepressant-like effect of ketamine in the LPS depression model, mice received a pretreatment with 3 sub-anaesthetic doses of ketamine: 1mg/Kg, 3mg/Kg and 10mg/Kg intraperitoneally, 1h before LPS injection.

The control group received the same volume of saline. 25h after this treatment (24h after LPS) the animals were submitted to the Open Field Test and modified Forced Swim Test.

### 2.9.2 Experiment 2

Buprenorphine hydrochloride was freshly prepared in experimental day. The compound was dissolved in sterile molecular grade deionized water and administered at 0.25 mg/kg intraperitoneally. Vehicle groups receveid distilled water in an equivalent volume (vehicle). Animals were submitted to Open Field and Forced Swim Test at the same time-point as in experiment 1. In a previous study, buprenorphine was tested in the Wistar Kyoto rat strain, a rodent model of exaggerated depressive and anxiety behaviors (BROWNE; NEST; LUCKI, 2015). However, to our knowledge this is the first study to investigate the effects of buprenorphine in the LPS depression model, which is associated with a challenge to the immune system.

## 3 Results

### Experiment 1

#### Sickness Behavior (8h)

Two-way ANOVA showed an effect of LPS [$F(1, 88) = 212.9$; $P<0.0001$] and an interaction between LPS ande Ketamine [$F(3, 88) = 3.84$; $P=0.0124$] . Tukey's *post hoc* test revealed that all groups that received LPS exhibited higher behavioral scores compared to saline-saline groups ($p\leq0.0001$). The group who received LPS and 10mg of ketamine presented a slightly attenutation of sickness behavior when compared to LPS/saline group ($p\leq0.003$). There was no effect of Ketamine [$F(3, 88) = 2.183$; n.s] (Fig 2a).

#### Temperature (8h*)*

Two-way ANOVA revealed an effect of LPS [F (1, 87) = 28.56; P<0.0001]. *Post hoc* test showed that all groups that received LPS presented hypothermia compared to saline group (p≤0.001). There was no effect of ketamine [F (3, 87) = 0.2263; n.s.] and no interaction between both factors [F (3, 87) = 1.156; n.s.] (Fig 2b).

Nesting Behavior

Regarding nesting behavior during 8h after LPS injection, two-way ANOVA showed an effect of LPS [F (1, 89) = 22.81; P<0.0001]. Further analyses revealed that all LPS-injected animals presented a decrease in nesting behavior (p≤0.05). There was no effect of ketamine [F (3, 89) = 0.118; n.s.] and no interaction between the factors [F (3, 89) = 0.03949; n.s.] (Fig 2c).

For nesting behavior 24h after LPS injection, two-way ANOVA showed an effect of LPS [F (1, 88) = 36.82; P<0,0001] and *post hoc* test revealed that LPS decreased the nesting behavior in all groups (p≤0.001). There was no effect of ketamine [F (3, 88) = 1.544; P=0.2089] and no interaction between LPS and Ketamine [F (3, 88) = 0.3926; P=0.7586] (Fig 2d).

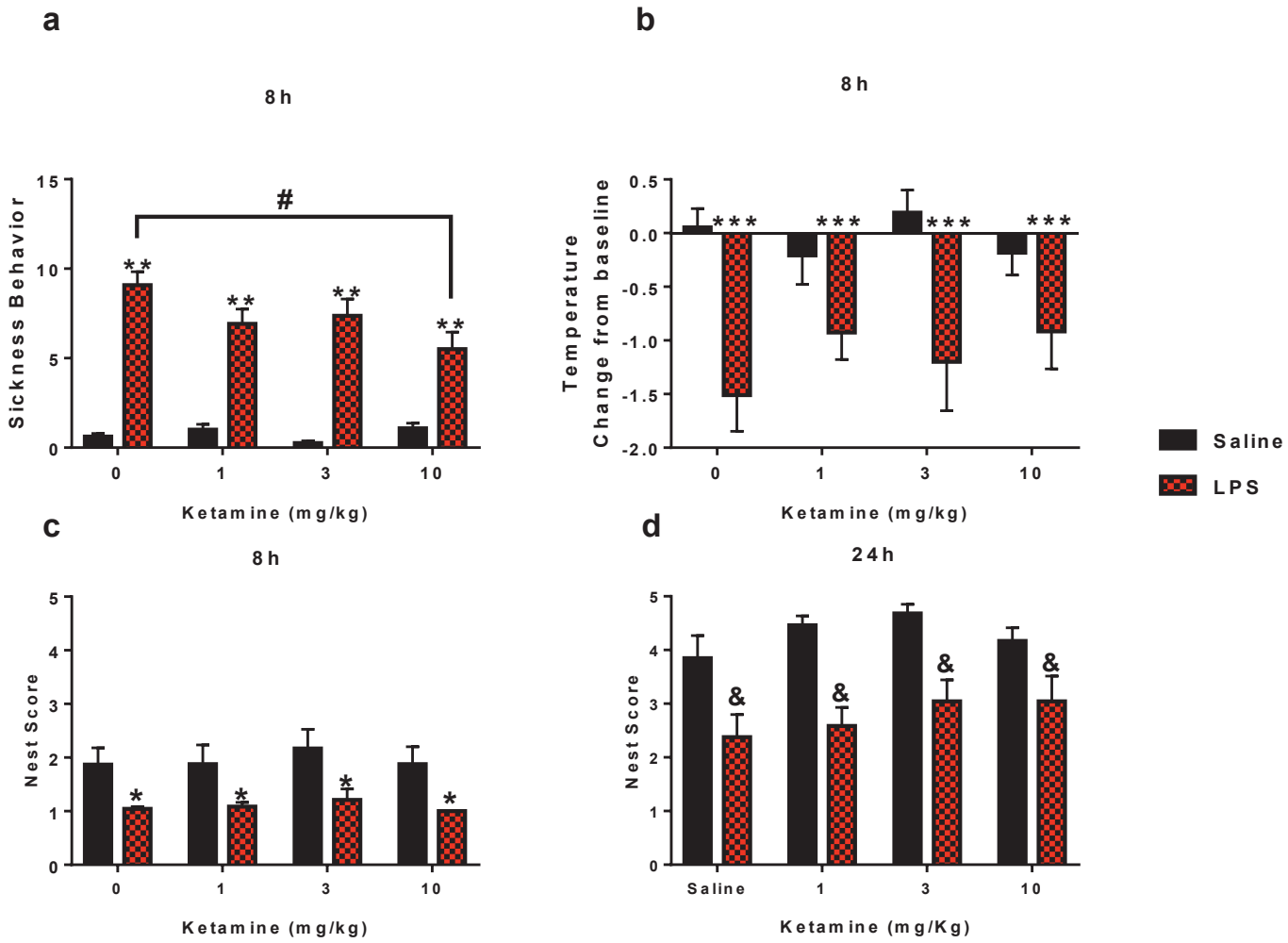


Fig 2 a Sickness behavior (behavioral scores). b Temperature (change from baseline). c Nesting behavior (nest score) 8h after LPS injection. d Nesting behavior (nest score) 24h after LPS injection. Two-way ANOVA followed by Tukey's post hoc test. **p≤0.0001 #p≤0.003 ***p≤0.001 *p≤0.05 &p≤0.001. Values are expressed by mean ± sem. n=11-13

## Pica Behavior

For kaolin intake 8h after LPS injection, two-way ANOVA revealed an effect of ketamine [F (3, 75) = 5.627; P=0.0016] and an interaction between ketamine and LPS [F (3, 75) = 5.165; p=0.0027]. *Post hoc* test showed that LPS increased kaolin intake compared to saline/saline group (p≤0.02). Ketamine at doses of 1mg and10mg completly blocked LPS-induced kaolin intake (p≤0.03 and p≤0.0008, respectively).

Two-way ANOVA did not show any effect of LPS [F (1, 75) = 0.4852; n.s.] (Fig 3a).

Regarding kaolin intake 24h after LPS injection, two-way ANOVA did not show any effect of LPS [F (1, 81) = 0.9352; n.s.], Ketamine [F (3, 81) = 0.9129; n.s.] and no interaction between these factors F (3, 81) = 1.486; n.s.] (Fig 3b).

Chow intake

Figure 3c shows chow intake 8h after LPS injection. Two-way ANOVA revealed an effect of LPS [F (1, 88) = 10.15; P=0.0020]. Further analyses revealed that all LPS groups presented a decrease in chow intake, including LPS-ketamine groups, compared to the respectives saline groups ($p \leq 0.05$).

For chow intake 24h after LPS, two-way ANOVA showed an effect of ketamine [F(3,82)= 3.259; p=0.0257] and an interaction between ketamine and LPS [F (3,82) = 3.51; $p \leq 0.02$]. *Post hoc* test showed that the LPS/Ketamine 1mg group presented less chow intake compared to all the others LPS-groups ($p \leq 0.01$). There was no effect of LPS [F (1, 82) = 2.962; n.s.] (Fig 3d).

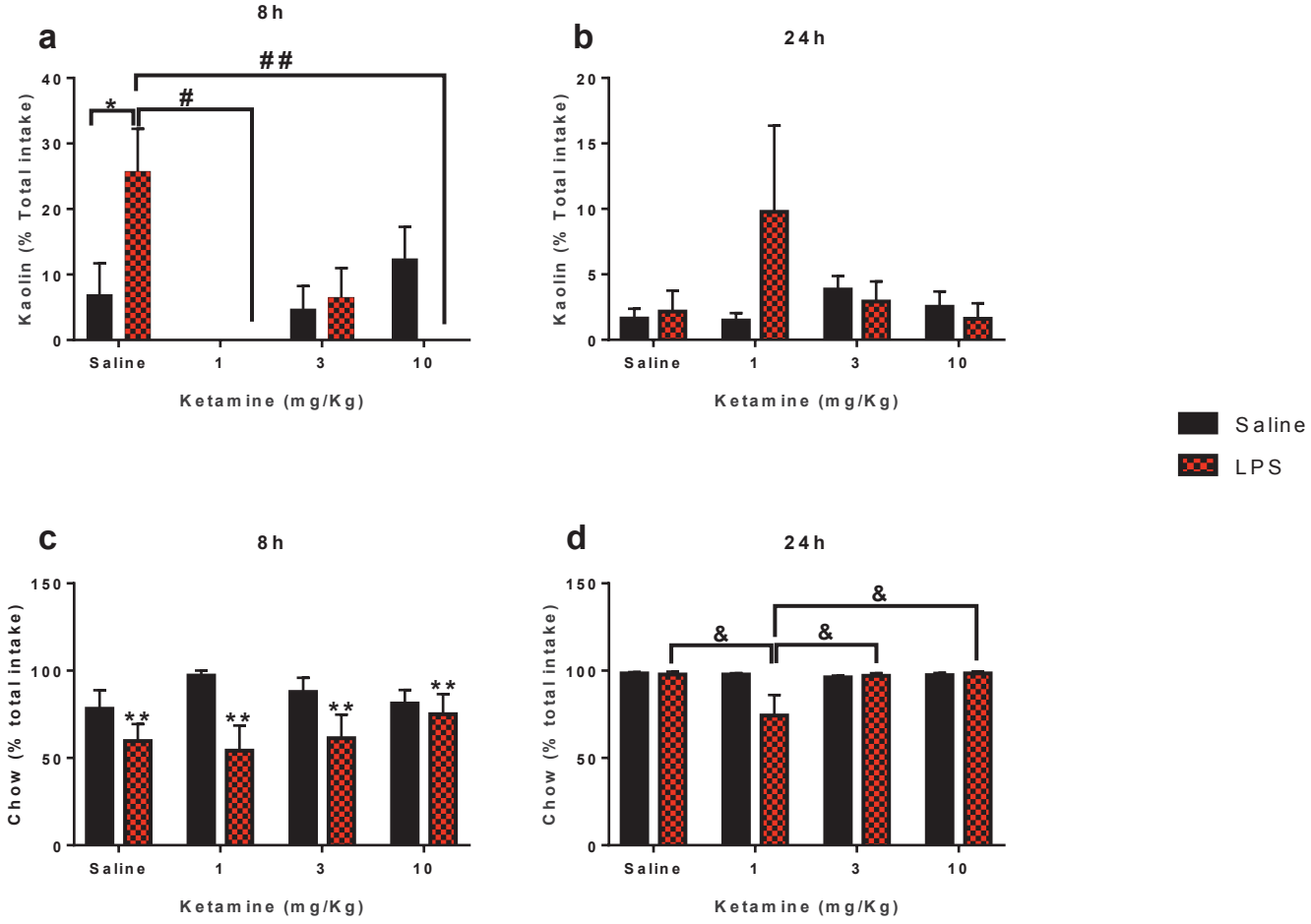


Fig 3 a Kaolin intake 8h after LPS injection. b Kaolin intake 24h after LPS injection. c chow intake 8h after LPS injection. d chow intake 24h after LPS injection. *p≤0.02 #p≤0.03 ##p≤0.0008 **p≤0.05 &p≤0.01. Two-way ANOVA followed by Tukey's *post hoc* test. Values are expressed as mean±sem. n = 11-14

Open Field

Regarding center time, two-way ANOVA did not show any effect of LPS [F (1, 82) = 0.6363; n.s.], ketamine [F (3, 82) = 0.99; n.s.] and no interaction between these factors [F (3, 82) = 0.712; n.s.] (Fig 4a).

For peripheral time, two-way ANOVA revealed an effect of ketamine [F (3, 80) = 2.84; n.s.] and na interaction between ketamine and LPS [F(3,80) = 3.396; n.s.]. Further analyses did not show any difference among groups. There was no effect of LPS [F (1, 80) = 0.01548; n.s.] (Fig 4b).

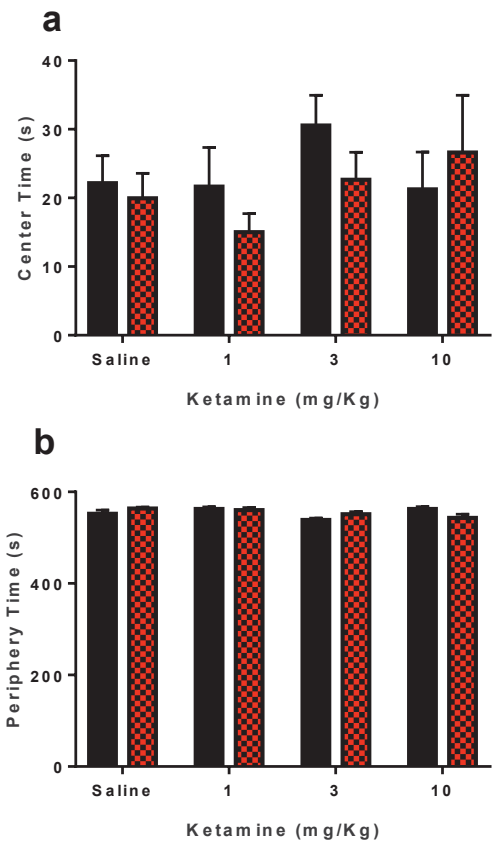


Fig 4 Open Field Test. a Center time (s) b Peripheral time (s). Two-way ANOVA. Values are expressed as mean ± sem. n = 11-14

## Sucrose preference test

For sucrose preference, two-way ANOVA showed an effect of LPS [F(1, 65) = 6.48; P=0.0133] and an interaction between LPS and ketamine [F(3,65) = 2.769; P=0.0486]. *Post hoc* test showed that LPS/saline group presented less sucrose intake compared to saline/saline group (p≤0.05) (5a). There was no effect of ketamine [F (3, 65) = 1.348; n.s.]. For water intake, there was an effect of LPS [F (1, 62) = 12.19; P=0.0009]. *Post hoc* test revealed that LPS increased water intake compared to all other groups (p≤0.03). There was no effect of of ketamine [F (3,62) = 2.357; n.s.] and no interaction between these factors [F (3, 62) = 2.046; n.s.] (5b).

Forced swim test

Two-way ANOVA revealed an interaction between LPS and ketamine [F (3,79) = 2.802; P=0.0452]. Follow-up analyses showed that LPS/saline group presented more immobility time when compared to the LPS group who received 10mg of ketamine (p≤0.05). There was no effect of LPS [F (1, 79) = 0.6285; n.s.] or ketamine [F (3, 79) = 0.9904; n.s.] (Fig 5c).

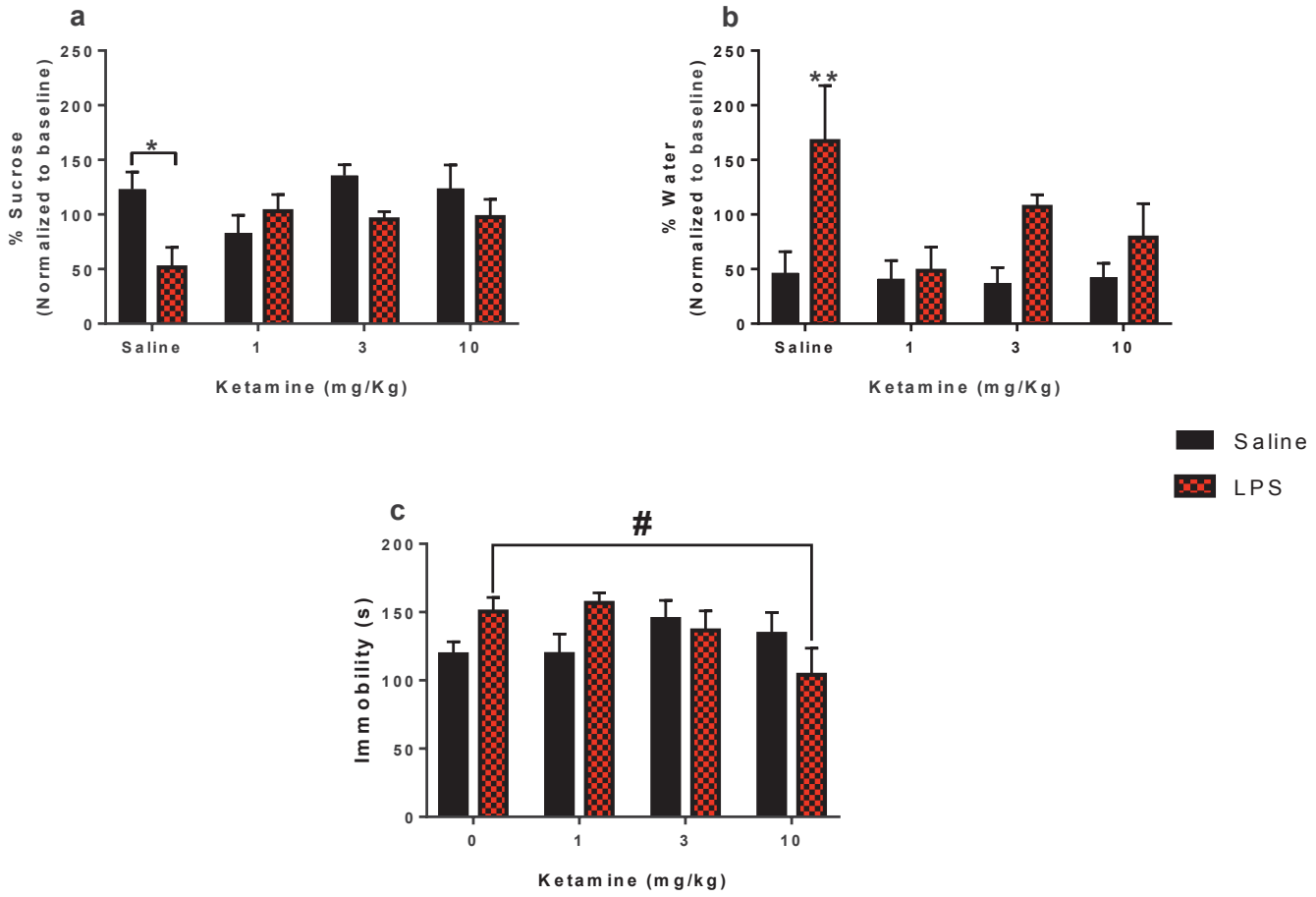


Fig 5 Sucrose preference test and forced swim test. a sucrose (% intake normalized to baseline). b water intake (% intake normalized to baseline). c Forced Swim test. *p≤0.05 **p ≤0.03 #p≤0.05. Two-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean ± sem.n=8 -12.

**Experiment 2**

Sickness Behavior (8h)

Two-way ANOVA showed an effect of LPS [F (1, 36) = 61.68; p<0.0001], an effect of buprenorphine [F (1, 36) = 8.156; p=0.0071] and an interaction between LPS and buprenorphine [F (1, 36) = 5.384; p=0.0261]. *Post hoc* test revealed that both LPS groups (LPS/Vehicle and LPS/BUP) presented higher behavioral scores compared to both saline groups (p≤0.0001). Buprenorphine attenuated sickness, seen by lower scores presented by this group compared to LPS/vehicle animals (p≤0.0043) (Fig 6a).

Temperature (8h)

Two-way ANOVA showed an effect of LPS [F (1, 34) = 13.65; P=0.0008] and buprenorphine [F (1, 34) = 17.37; P=0.0002]. Further analyses revealed that LPS induced a decrease of body temperature, compared to saline/vehicle (p≤0.01) and saline/buprenorphine animals (p≤0.0001). Buprenorphine pretreament prevented LPS-induced hypothermia (p≤0.007). There was no interaction between LPS and buprenorphine [F (1, 34) = 0.7851; n.s.) (6b).

Nesting behavior

For nesting behavior 8h after LPS injection, two-way ANOVA an effect of LPS [F (1, 36) = 21.97; p<0.0001]. *Post hoc* test showed that LPS decreased nesting behavior compared to saline/vehicle animals (LPS/vehicle and LPS/buprenorphine vs saline/vehicle; p≤0.0004). There was no effect of buprenorphine [F (1, 36) = 2.612; n.s.] and no interaction between LPS and buprenorphine [F (1, 36) = 2.612; n.s.] (6c).

For nesting behavior 24h after LPS, there was an effect of LPS [F (1, 36) = 73.65; P<0.0001] and an effect of buprenorphine [F (1, 36) = 5.101; P=0.0301]. *Post hoc* test showed that all animals that received LPS presented less nesting behavior compared to both saline groups (p≤0.0004.) There was no interaction between LPS and buprenorphine [F (1, 36) = 0.204; n.s.] (6d).

Pica behavior

For kaolin intake 24h after LPS, there was no effect of LPS [F (1, 33) = 0.7756; n.s.], buprenorphine [F (1, 33) = 2.655; n.s.] and no interaction between these factors [F (1, 33) = 1.022; n.s.] (Fig 6e).

Chow intake

Two-way ANOVA did not show any effect of LPS [F (1, 33) = 0.7756; n.s.], buprenorphine [F (1, 33) = 2.655; n.s.] and no interaction between these factors [F (1, 33) = 1.022; n.s.] (Fig 6f).

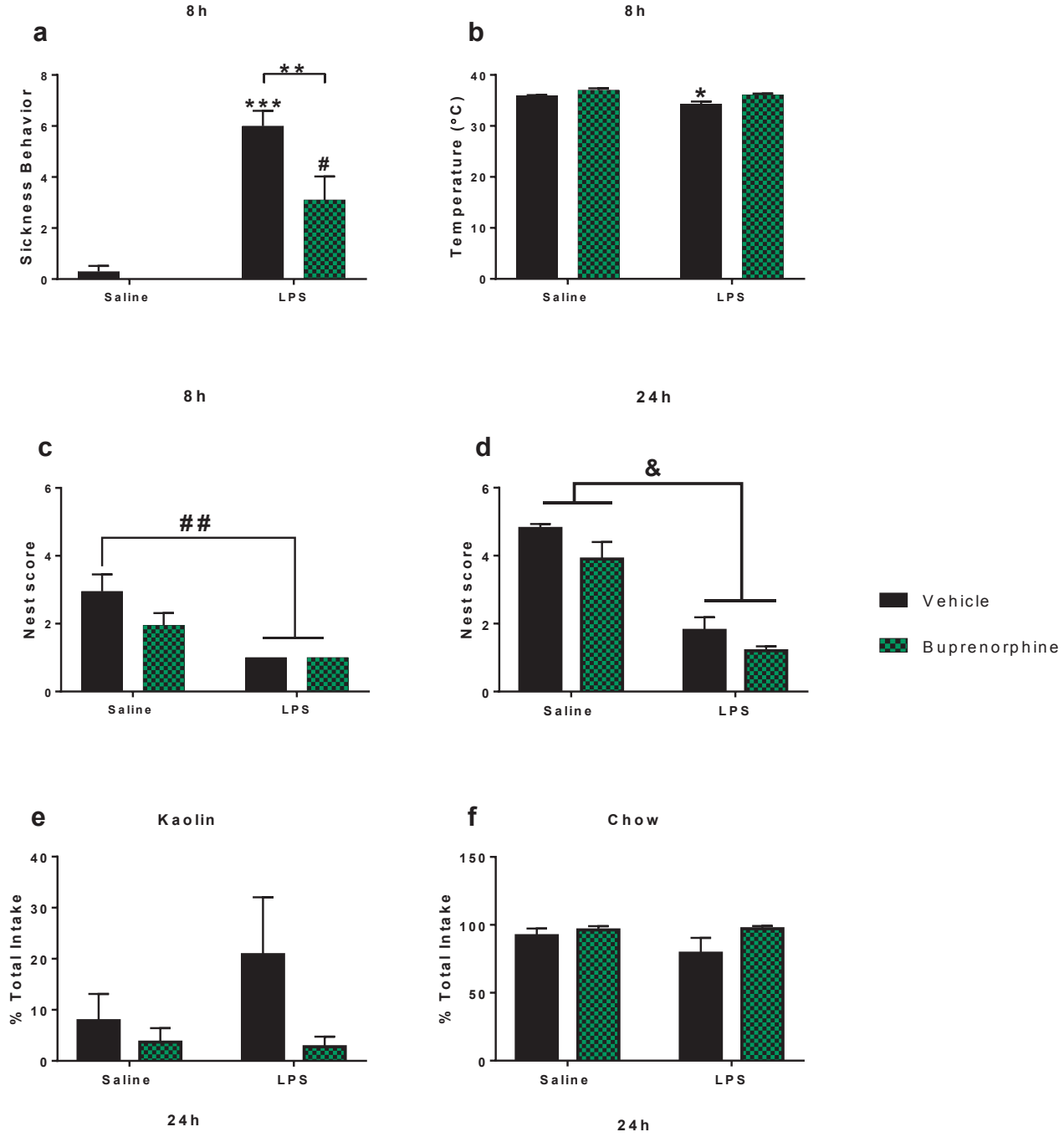


Fig 6 a) Sickness behavior b) temperature c) Nesting Behavior 8h after LPS d) Nesting Behavior 24h after LPS e) Kaolin intake 24h after LPS f) Chow intake 24h after LPS. Two-way ANOVA followed by Tukey's *post Hoc* test. ***p≤0.0001 compared to both saline groups. #p≤0.006 compared to saline groups. **p≤0.004. *p≤0.01. ##p≤0.0004 &p≤0.0004. n=9 -10.

## Open Field test

Regarding center time, there was an effect of LPS [F (1, 34) = 6.612; n.s.]. Further analyses revealed that both LPS groups – LPS/vehicle and LPS/buprenorphine

– presented less time in the center of arena compared to both saline groups (p≤0.05). There was no effect of buprenorphine [F (1, 34) = 0.07205; n.s.] and no interaction between LPS and buprenorphine [F (1, 34) = 0.06636; n.s.] (Fig 7a).

For peripheral time, two-way ANOVA showed an effect of LPS [F (1, 34) = 8.599; P=0.0060]. *Post hoc* test showed that both LPS groups remained more time in perihery compared to both saline groups (p≤0.05). There was no effect of buprenorphine [F (1, 34) = 3.102; n.s.] and no interaction between LPS and buprenorphine [F (1, 34) = 0.07746; n.s.] (7b).

Sucrose preference test

Two-way ANOVA showed an effect of LPS [F (1, 33) = 5.53; P=0.0248]. Follow-up analyses revealed that both LPS groups (LPS/vehicle and LPS/Buprenorphine) presented less sucrose intake compared to saline groups (p≤0.05). There was no effect of buprenorphine [F (1, 33) = 0.8639; n.s.] and no interaction [F (1, 33) = 0.5794; n.s.] (7c). For water intake, there was an effect of LPS [F (1, 34) = 6.7; P=0.0141]. *Post hoc* test showed that both LPS groups drank more water compared to both saline groups (p≤0.05). There was no effect of buprenorphine [F (1, 34) = 0.4117; n.s.] and no interaction [F (1, 34) = 0.2266; n.s.] (7d).

Forced swim test

For immobility time, there was an effect of LPS [F (1, 31) = 4.531; P=0.0413] and an interaction between LPS and buprenorphine [F (1, 31) = 4.693; P=0.0381]. *Post hoc* test showed that LPS/buprenorphine group presented less immobility time compared to all other groups (p≤0.05). There was no effect of buprenorphine [F (1, 31) = 0.4729; n.s.] (7e).

For latency to immobility, two-way ANOVA showed an effect of LPS [F (1, 31) = 13.14; P=0.0010] and an interaction between LPS and buprenorphine [F (1, 31) = 5.702; P=0.0232]. *Post hoc* test showed that LPS/vehicle group presented less time to became immobile compared to all other groups (p≤0.04) (7f).

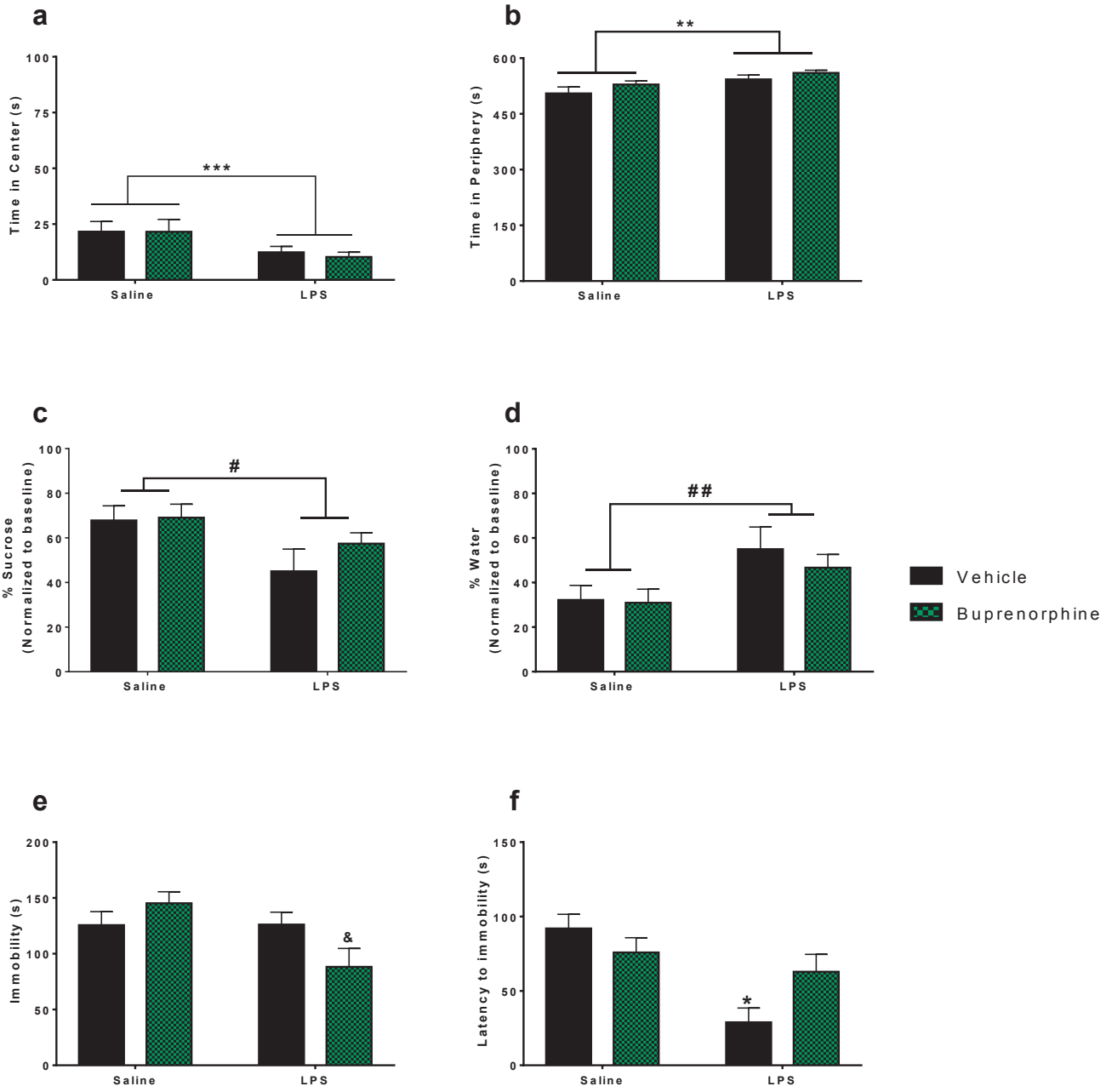


Fig 7. Open field test: a center time b peripheral time. Sucrose preference test: c sucrose intake d water intake. Forced swim test: e immobility time f latency to immobility. ***p≤0.05 **p ≤0.05 #p≤0.05 ##p≤0.05 &p≤0.05 *p≤0.04. Two-way ANOVA followed by Tukey's *post hoc* test. Values are expressed as mean ± sem. n= 8-10.

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